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Protective Actions of Oxytocin on Aortic Wistar and SHR VSMCs

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PROTECTIVE ACTIONS OF OXYTOCIN ON AORTIC WISTAR AND SHR VSMCS

By

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This is to certify that the Ph.D. Dissertation of

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With a major in Pharmaceutical Sciences with a specialization in Molecular Medicine and Pharmacogenomics has been approved by the Examining Committee on May 31, 2019 as satisfactory for the dissertation requirement for the Doctor of Philosophy degree.

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Abstract

An Abstract of a Dissertation Submitted to Nova Southeastern University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

PROTECTIVE ACTIONS OF OXYTOCIN ON AORTIC WISTAR AND SHR VSMCS

By

VARUN KUMAR SOTI

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Background. Oxytocin, known as a female reproductive hormone, has emerged as a cardiovascular hormone showing cardioprotective effects, for instance, negative chronotropic and inotropic effects and ANP release in cardiomyocytes and endothelium. However, oxytocin's effect in VSMCs remains to be explored. **Objectives.** This study had three goals: characterizing oxytocin's effect on proliferation, inflammation, oxidative stress, and vasocontraction pathway in Wistar VSMCs; determining oxytocin's effect on Ang II; describing oxytocin's effect and its interaction with Ang II in SHR VSMCs. **Methods:** Aortic VSMCs were isolated from Wistar rats and SHRs. Hemocytometer, MTT and ^{3}H-Thymidine incorporation assays, and flow cytometry were employed for cell proliferation studies. Two key proliferation pathways — ERK1/2 and PI3K/Akt pathways, and vasocontraction (ROCK) pathway were investigated for mechanistic studies utilizing Western blotting. IL-6 and ROS were measured to assess inflammation and oxidative stress, respectively. **Results.** Cell proliferation studies and Western blotting outcomes in Wistar and SHR VSMCs, demonstrated that oxytocin (100 nM) significantly decreased cell proliferation and reduced phosphorylation of ERK1/2, PI3Kp110α, Akt, ROCK-1, and ROCK-2, respectively. OXTA antagonized these effects of oxytocin. Flow cytometric measurements indicated that oxytocin-induced apoptosis and necrosis. IL-6 assay and ROS assay results displayed that oxytocin increased IL-6 and ROS, respectively. Oxytocin's pre-treatment with Ang II in Wistar and SHR VSMCs caused: decreased cell proliferation; reduced phospho-ERK1/2, phospho-PI3Kp110α, phospho-Akt, ROCK-1, and ROCK-2; decreased IL-6; increased ROS. Comparison between Wistar and SHR VSMCs illustrated that oxytocin greatly reduced PI3Kp110α phosphorylation in Wistar VSMCs than SHR VSMCs ($P < 0.01$). Oxytocin caused higher IL-6 secretion in SHR VSMCs ($P < 0.01$) and higher ROS production in Wistar VSMCs ($P < 0.001$). Oxytocin's pre-treatment with Ang II showed: higher reduction in ERK1/2 phosphorylation in SHR VSMCs ($P < 0.001$); higher reduction in PI3Kp110α phosphorylation ($P < 0.05$) and higher increase in ROS in Wistar VSMCs ($P < 0.001$). **Conclusion.** Oxytocin showed an anti-proliferative effect on aortic VSMCs, likely through inhibition of ERK1/2 and PI3K/Akt phosphorylation. Oxytocin-mediated decreased phosphorylation of ROCK-1 and ROCK-2 suggest oxytocin's anti-vasoconstrictive action in VSMCs. Oxytocin's antagonistic actions against Ang II indicate that oxytocin may exert beneficial vascular effects in conditions associated with high levels of Ang II.

Dedication

This is dedicated:

To the late Mrs. Chandrawati Devi, my beloved Grandmother. I miss you every second of my life;

To my greatest inspiration, Mrs. Sushma Sharma, my loving Mother;

To the strongest man I know, Dr. Gopal Krishna Soti, my dear Father;

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Table of Contents

Abstract	iii
Dedication	iv
Acknowledgements	v
Table of Contents	viii
List of Figures	xi
List of Tables	xiv
List of Abbreviations	xv
Chapter 1. Introduction	1
1.1. Background	1
1.2. Traditional Roles of Oxytocin	4
1.3. Emerging Role of Oxytocin in the CVS	5
1.3.1. Effect of Oxytocin on Heart	5
1.3.2. Effect of Oxytocin on Cardiomyocytes	6
1.3.3. Effect of Oxytocin on Blood Vessels (ECs and VSMCs)	8
1.3.4. Effect of Oxytocin on Pathophysiological conditions of CVS	10
1.4. Role of Ang II in the CVS and its Pathophysiology	12
1.5. The Gap in the Literature, Research Hypotheses, and Study Aims	15
1.5.1. Hypothesis 1	15
1.5.2. Hypothesis 2	16
1.5.3. Specific Aim 1	16
1.5.4. Specific Aim 2	16
1.5.5. Specific Aim 3	17
1.6. Role of VSMCs in the CVS and its Pathophysiology	18
1.7. Study Significance	21
1.8. Barriers, Issues, Limitations, and Delimitations	22
Chapter 2. Literature Review	24
2.1. MAPK/ERK1/2 Pathway in the CVS	24
2.2. PI3K/Akt Signaling in the CVS	29
2.3. Rho Kinases (ROCK-1 and ROCK-2) Signaling in the CVS	35
2.4. Interleukin-6 in the CVS	41
2.5. Reactive oxygen species (ROS) in the CVS	44
2.6. Summary	48
Chapter 3. Methodology	49
3.1. Wistar rats and Spontaneously Hypertensive Rats (SHRs)	49
3.2. Isolation and Culture of Primary Rat Aortic VSMCs	49
3.3. VSMCs Proliferation Assessment by the Hemocytometer Method	51
3.3.1. The Principle of the Hemocytometer Method	51
3.3.2. The Procedure of Cell Counting by the Hemocytometer Method	52
3.4. VSMCs viability determination by the MTT Assay	53

3.4.1. The Principle of the MTT Assay 53
3.4.2. The Procedure of the MTT Assay 53
3.5. VSMCs Growth Measurement by the ^{3}H-Thymidine Incorporation Assay 54
3.5.1. The Principle of the ^{3}H-Thymidine Incorporation Assay 54
3.5.2. The Procedure of the ^{3}H-Thymidine Incorporation Assay 54
3.6. VSMCs Apoptosis and Necrosis Measurement by Flow Cytometery 56
3.6.1. The Principle of Flow Cytometery 56
3.6.2. The Procedure of Flow Cytometery 59
3.7. IL-6 Measurement by the ELISA 61
3.7.1. The Principle of IL-6 ELISA 61
3.7.2. The Procedure of IL-6 ELISA 63
3.8. ROS Assay 64
3.8.1. The Principle of ROS Assay 64
3.8.2. ROS Assay Procedure 64
3.9. Western Blotting 66
3.9.1. The Principle of Western Blotting 66
3.9.2. Preparation of Cell Lysate and Procedure of Western Blotting 66
3.10. Statistical Analysis 74
3.11. Specific Aim 1 and 2, Strategies, and Experimental Design 75
3.12. Specific Aim 3, Strategies, and Experimental Design 82

Chapter 4. Results 83

4.1. Normotensive Wistar Rats (Specific Aims 1 and 2) 83
4.1.1. Effects of oxytocin and Ang II on Proliferation of Aortic Wistar VSMCs 83
4.1.1.1. Cell counting by the Hemocytometer Method in Wistar VSMCs 83
4.1.1.2. Assessment of Cell Viability with MTT-assay in Wistar VSMCs 87
4.1.1.3. Cell Growth Assessment with ^{3}H-Thymidine Incorporation assay in Wistar VSMCs 90
4.1.1.4. Assessment of Cell Viability by Flow Cytometry in Wistar VSMCs 92
4.1.2. Effect of Oxytocin and its Pre-treatment with Ang II on the Phosphorylation of ERK1/2 in Aortic Wistar VSMCs 96
4.1.3. Effect of Oxytocin and its Pre-treatment with Ang II on the Phosphorylation of PI3K p110a and Akt in Aortic Wistar VSMCs 98
4.1.4. Effect of Oxytocin and its Pre-treatment with Ang II on the Activation of ROCK -1 and ROCK-2 in aortic Wistar VSMCs 101
4.1.5. Effect of Oxytocin and its Pre-treatment with Ang II on IL-6 secretion in Aortic Wistar VSMCs 105
4.1.6. Effect of Oxytocin and its Pre-treatment with Ang II on ROS in Aortic Wistar VSMCs 108
4.2. Spontaneously Hypertensive Rats (Specific Aim 3) 111
4.2.1. Effect of Oxytocin and its Pre-treatment with Ang II on Aortic SHR VSMCs proliferation 111
4.2.1.1. Cell counting by the Hemocytometer method in SHR VSMCs 111
4.2.1.2. Assessment of Cell Viability with MTT-assay in SHR VSMCs 114

4.2.1.3. Cell Growth Assessment with ^{3}H-Thymidine Incorporation Assay in SHR VSMCs 117
4.2.1.4. Assessment of Cell Viability by Flow Cytometry in SHR VSMCs 119
4.2.2. Effect of Oxytocin and its Pre-treatment with Ang II on the Phosphorylation of ERK1/2 in Aortic SHR VSMCs 122
4.2.3. Effect of Oxytocin and its Pre-treatment with Ang II on the Phosphorylation of PI3K p110α and Akt in Aortic SHRs VSMCs 124
4.2.4. Effect of Oxytocin and its Pre-treatment with Ang II on the Activation of ROCK -1 and ROCK-2 in aortic SHR VSMCs 127
4.2.5. Effect of Oxytocin and its Pre-treatment with on IL-6 secretion in Aortic SHR VSMCs 130
4.2.6. Effect of Oxytocin and its Pre-treatment with Ang II on ROS production in Aortic SHR VSMCs 133
4.3. Comparison of the Effects of Oxytocin and its Pre-treatment with Ang II on aortic Wistar VSMCs versus SHR VSMCs 136
4.3.1. Comparison of the Effect of Oxytocin and its Pre-treatment with on cell proliferation in Aortic Wistar VSMCs and SHR VSMCs 136
4.3.2. Comparison of the Effect of Oxytocin and its Pre-treatment with Ang II on Cell Viability in Aortic Wistar VSMCs and SHR VSMCs 139
4.3.3. Comparison of the Effect of Oxytocin on Apoptosis and Necrosis in Aortic Wistar VSMCs between and SHR VSMCs 142
4.3.4. Comparison of the Effect of Oxytocin and its Pre-treatment with Ang II on the Phosphorylation of ERK1/2, PI3K p110a, and AKT in Aortic Wistar VSMCs and SHR VSMCs 146
4.3.5. Comparison of the Effect of Oxytocin and its Pre-treatment with Ang II on Vasocontraction Pathway: ROCK-1 and ROCK-2 in Aortic VSMCs Isolated from Wistar rats and SHRs 150
4.3.6. Comparison of the Effect of Oxytocin and its Pre-treatment with Ang II on IL-6 in Aortic Wistar VSMCs and SHR VSMCs 153
4.3.7. Comparison of the Effect of Oxytocin and its Pre-treatment with Ang II on ROS production in Aortic Wistar VSMCs and SHR VSMCs 157

Chapter 5. Discussion 159

5.1. Oxytocin Reduced the Proliferation in Aortic VSMCs 162
5.2. Oxytocin Deactivated the Proliferation Signal Transduction Pathways (ERK1/2 Pathway and PI3K/Akt Pathway) in Aortic VSMCs 164
5.3. Oxytocin Deactivated the ROCK Pathway in Aortic VSMCs 168
5.4. Oxytocin Increased IL-6 in Aortic VSMCs 171
5.5. Oxytocin Increased ROS in Aortic VSMCs 174
5.6. Summary of the Dissertation Research 177
5.7. Conclusion 181
5.8. Future recommendation 182

Bibliography 184

List of Figures

Figure 1	Histology of a Blood Vessel	18
Figure 2.1	Activation of MAPK Signaling Pathway in the CVS	25
Figure 2.2	The PI3K/Akt Signaling Pathway in the CVS	31
Figure 2.3	ROCK Signaling Pathway in the Contraction of VSMCs	40
Figure 2.4	Redox Signaling in the CVS	46
Figure 3.1	Hemocytometer and the Grid lines of a Chamber in a Hemocytometer Microscopic Slide	51
Figure 3.2	Flow Cytometer	57
Figure 3.3	Presentation of Markers for Detection by Healthy, Apoptotic, Late-Apoptotic and Necrotic Cells	58
Figure 3.4	A Typical ELISA Sandwich	62
Figure 3.5	A Schematic Representation of the Various Steps in Western Blotting	68
Figure 4.1	The Effect of Oxytocin and Different Treatment Groups on Aortic VSMCs Proliferation in Wistar rats as Assessed by the Hemocytometer Method	85
Figure 4.2	The Effect of Oxytocin and Different Treatment Groups on aortic VSMCs Proliferation Rate of Aortic VSMCs in Wistar rats as Assessed by the Hemocytometer Method	86
Figure 4.3	The Effect of Oxytocin and Different Treatment Groups on Wistar aortic VSMCs Viability as Assessed by the MTT Assay	88
Figure 4.4	The Effect of Oxytocin and Different Treatment Groups on Wistar aortic VSMCs Viability Rate as Assessed by the MTT Assay	89
Figure 4.5	The Effect of Oxytocin and Different Treatment Groups on Wistar aortic VSMCs Growth as Assessed by the ^{3}H-Thymidine Incorporation Assay	91
Figure 4.6	The Effect of Oxytocin on Apoptosis and Necrosis in Aortic Wistar VSMCs as Assessed by Flow Cytometry	94
Figure 4.7	The Effect of Oxytocin and Different Treatment Groups on the Phosphorylation of ERK1/2 in Aortic Wistar VSMCs	97
Figure 4.8	The Effect of Oxytocin and Different Treatment Groups on the Phosphorylation of PI3K p110α in Aortic Wistar VSMCs	99
Figure 4.9	The Effect of Oxytocin and Different Treatment Groups on the Phosphorylation of Akt in Aortic Wistar VSMCs	100
Figure 4.10	The Effect of Oxytocin and Different Treatment Groups on the Activation of ROCK- 1 in Aortic Wistar VSMCs	102
Figure 4.11	The Effect of Oxytocin and Different Treatment Groups on the Activation of ROCK- 2 in Aortic Wistar VSMCs	103
Figure 4.12	The Effect of Oxytocin and Different Treatment Groups on IL-6 Secretion in Aortic Wistar VSMCs	107
Figure 4.13	The Effect of Oxytocin and Different Treatment Groups on ROS Production in Aortic Wistar VSMCs	110

Figure 4.14	The Effect of Oxytocin and Different Treatment Groups on Aortic VSMCs Proliferation in SHRs as Assessed by the Hemocytometer Method	112
Figure 4.15	The Effect of Oxytocin and Different Treatment Groups on the Proliferation Rate of Aortic VSMCs in SHRs as assessed by the Hemocytometer method	113
Figure 4.16	The Effect of Oxytocin and Different Treatment Groups on Aortic SHR VSMCs Viability as Assessed by the MTT assay	115
Figure 4.17	The Effect of Oxytocin and Different Treatment Groups on Aortic SHR VSMCs Viability Rate as Assessed by the MTT assay	116
Figure 4.18	The Effect of Oxytocin and Different Treatment Groups on Aortic SHR VSMCs Growth as Assessed by the ^{3}H-Thymidine Incorporation Assay	118
Figure 4.19	The Effect of Oxytocin on Apoptosis and Necrosis in aortic SHR VSMCs as Assessed by Flow Cytometry	120
Figure 4.20	The Effect of Oxytocin and Different Treatment Groups on the Phosphorylation of ERK1/2 in Aortic SHR VSMCs	123
Figure 4.21	The Effect of Oxytocin and Different Treatment Groups on the Phosphorylation of PI3K p110α in Aortic SHR VSMCs	125
Figure 4.22	The Effect of Oxytocin and Different Treatment Groups on the Phosphorylation of Akt in Aortic SHR VSMCs	126
Figure 4.23	The Effect of Oxytocin and Different Treatment Groups on the Activation of ROCK- 1 in Aortic SHR VSMCs	128
Figure 4.24	The Effect of Oxytocin and Different Treatment Groups on the Activation of ROCK- 2 in Aortic SHR VSMCs	129
Figure 4.25	The Effect of Oxytocin and Different Treatment Groups on IL-6 Secretion in Aortic SHR VSMCs	132
Figure 4.26	The Effect of Oxytocin and Different Treatment Groups ROS Production in aortic SHR VSMCs	135
Figure 4.27	The Comparison of the Effects of Oxytocin, Ang II, and the Pre-treatment of Oxytocin with Ang II on Proliferation of Aortic VSMCs Between Wistar VSMCs and SHR VSMCs as Assessed by the Hemocytometer Method	138
Figure 4.28	The Comparison of the Effects of Oxytocin, Ang II, and the Pre-treatment of Oxytocin with Ang II on Viability of Aortic VSMCs Between Wistar VSMCs and SHR VSMCs as Assessed by the MTT Assay	141
Figure 4.29	The Comparison of the Effects of Oxytocin on Live cells, Apoptosis, and Necrosis Between Wistar VSMCs and SHR VSMCs as Assessed by the Flow Cytometry	144
Figure 4.30	The Comparison of the Effects of Oxytocin, Ang II, and the Pre-treatment of Oxytocin with Ang II on the Phosphorylation of ERK1/2 (figure a, b, and c), PI3K p110α (figure d, e, and f), and Akt (figure g, h, and i) on Aortic VSMCs Between Wistar VSMCs and SHR VSMCs	149

Figure 4.31	The Comparison of the Effects of Oxytocin, Ang II, and the Pre-treatment of Oxytocin with Ang II on the Phosphorylation of ROCK-1 (figure a, b, and c) and ROCK-2 (figure d, e, and f) on Aortic VSMCs Between Wistar VSMCs and SHR VSMCs	152
Figure 4.32	The Comparison of the Effect of Oxytocin (10 nM, 100 nM, and 1000nM), Ang II (100 nM), Pre-treatment of Oxytocin with Ang II on IL-6 Secretion between Wistar VSMCs and SHR Aortic VSMCs	155
Figure 4.33	The Comparison of the Effect of Oxytocin (100 nM), Ang II (100 nM), Pre-treatment of Oxytocin (100 nM) with Ang II (100 nM) on IL-6 Secretion between Wistar VSMCs and SHR Aortic VSMCs	156
Figure 4.44	The Comparison of the Effect of Oxytocin, Ang II, Pre-treatment of Oxytocin with Ang II on ROS Production between Wistar VSMCs and SHR Aortic VSMCs	158
Figure 5	Inhibition of the ERK1/2 Pathway, the PI3K/Akt Pathway, and the Rho-kinase Pathway by Oxytocin in Aortic VSMCs	170

List of Tables

Table 3.1.	Primary and Secondary Antibodies used in Western Blotting	69
Table 3.2.	Concentrations of Primary and Secondary Antibodies used in Western Blotting	71
Table 3.3.	Composition of Buffers used in Western Blotting	72
Table 3.4.	Composition of 10% Resolving (Separating) Gel used in Western Blotting	73
Table 3.5.	Composition of 5% Stacking Gel used in Western Blotting	73
Table 3.6.	Treatment Groups in Hemocytometer Method, MTT, and ^{3}H-Thymidine Incorporation Assays	76
Table 3.7.	Treatment Groups for ERK1/2 and PI3K/Akt Pathway Experiments	77
Table 3.8.	Treatment Groups for the ROS Assay	78
Table 3.9.	Treatment Groups for the IL-6 Assay	79
Table 3.10.	Treatment Groups for Rho Kinase Pathway Experiments	80

List of Abbreviations

°C	Degree in Celsius
α1-ADR	Alpha-1 Adrenergic Receptors
^{3}H-Thymidine	Tritiated Thymidine (Radioactive Thymidine)
4E-BP1	4E-Binding Protein 1
7-AAD	7-Amino Actinomycin D
AC	Adenylyl Cyclase
ACE	Angiotensin Converting Enzyme
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of Variance
ANP	Atrial Natriuretic Peptide
AP-1	Activator Protein-1
APS	Ammonium Per Sulfate
AT-1R	Angiotensin Type 1 Receptors
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
BH_4	Tetrahydrobiopterin
cAMP	Cyclic Adenosine Monophosphate
Cat / CAT	Catalase
CMs	Cardiomyocytes
CNS	Central Nervous System
CO_2	Carbon Dioxide
CPI-17	C-Kinases Potentiated Protein Phosphatase 1 Inhibitor -17
cpm	Counts Per Minute
CRP	C Reactive Protein
CVD	Cardiovascular Diseases
CVS	Cardiovascular System
CXCL	Chemokine (C-X-C Motif) Ligand
DAG	1, 2-Diacylglycerol
DCF	2',7'–Dichlorofluorescein
DCFDA	2',7'– Dichlorofluorescein Diacetate
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
ECs	Endothelial Cells
eEF2	Eukaryotic Elongation Factor 2
eIF4	Eukaryotic Initiation Factor 4
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular Signal-Regulated Kinase
ET	Endothelin
FBS	Fetal Bovine Serum

FKBP	FK506-Binding Protein
g	Gram
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDI	Guanine Nucleotide Dissociation Inhibitor
GEF	Guanine Nucleotide Exchange Factor
GLUT	Glucose Transporter
gp130	Glycoprotein 130
GPCRs	G-Protein Coupled Receptors
GPx	Glutathione Peroxidase
GRx	Glutathione Reductase
GSH	Glutathione (Reduced)
GSK	Glycogen Synthase Kinase
GST	Glutathione S-Transferase
GTP	Guanosine Triphosphate
GTP	Guanosine-5'-Triphosphate
$G\alpha_i$	G Alpha Inhibitory Protein
h-CD	H-Caldesmon
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
HEK293	Human Embryonic Kidney
HO-1	Heme Oxygenase-1
HRP	Horseradish Peroxidase
HSPG	Heparan Sulfate Proteoglycan
ICAM-1	Intercellular Adhesion Molecule-1
IGF	Insulin-Like Growth Factor
IL-6	Interleukin-6
IL-6R	Interleukin -6 Receptor
IP3	Inositol-3-Phosphate
JNK	C-Jun N-Terminal Kinases
KCl	Potassium Chloride
K_D	Equilibrium Dissociation Constant
KD	Kilo Dalton
KH_2PO_4	Potassium Dihydrogen Phosphate
KLF2	Kruppel-Like Factor 2
L-VGCC	L-Type Voltage-Gated Calcium Channel
M	Molar
MAPK	Mitogen-Activated Protein Kinase
MAPKK	Mitogen-Activated Protein Kinase Kinase
MAPKKK	Mitogen-Activated Protein Kinase Kinase Kinase
MCP-1	Monocyte Chemoattractant Protein-1
MI	Myocardial Infarction
MIP-2α	Macrophage Inflammatory Protein-2 Alpha
mL	Milliliter
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatases
mm	Millimeter

mM	Micro Molar
MMP	Matrix Metalloproteinase
MnSOD	Manganese Superoxide Dismutase
MSCs	Mesenchymal Stem Cells
mTOR	Mammalian Target of Rapamycin
MTT	3-(4,5-Dimethylthiazo-2-Yl)-2,5-Diphenyl Tetrazolium Bromide
MYPT-1	Myosin Phosphatase Target Subunit-1
Na_2HPO_4	Disodium Hydrogen Phosphate
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
NE	Norepinephrine
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NK	Natural Killer
nm	Nano Meter
nM	Nano Molar
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
Nrf2	Nuclear Factor (Erythroid-Derived 2) -Like 2
NSU-IACUC	Nova Southeastern University Institutional Animal Care and Use Committee
O_2^-	Superoxide
OD	Optical Density
OXTA	Oxytocin Receptor Antagonist – L-371,257
OXTRG	Oxytocin Receptor Gene
P value	Probability Value
p70S6K	P70s6 Kinase
PAH	Pulmonary Arterial Hypertension
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate-Buffered Saline
PDK	Phosphoinositide-Dependent Kinase
pg	Picogram
Phospho-Akt	Phosphorylated Akt
Phospho-ERK1/2 or pERK	Phosphorylated ERK1/2
Phospho-PI3K	Phosphorylated PI3K
PI	Phosphatidylinositol
PI-3-P	Phosphatidylinositol-3-Phosphate
PI-3,4-P_2	Phosphatidylinositol-3,4-Biphosphate
PI3K	Phosphatidylinositol-3-Kinases
PIP_3	Phosphatidylinositol Triphosphate
PKB	Protein Kinase B
PKC	Protein Kinase C
PLC	Phospholipase C
PP2A	Protein Phosphatase 2 A

PS	Phosphatidylserine
PtdIns	Phosphatidylinositol
PTEN	Phosphatase and Tensin Homolog Deleted on Chromosome Ten
Pyk-2	Proline-Rich Tyrosine Kinase-2
RA	Rheumatoid Arthritis
RAAS	Renin-Angiotensin-Aldosterone System
RBD	Rho-Binding Domain
Rho-GEFs	Rho Guanine Nucleotide Exchange Factors
RNA	Ribonucleic Acid
ROCK	Rho-Associated Coiled-Coil Kinase
ROS	Reactive Oxygen Species
rpm	Rotation Per Minute
RSK	Ribosomal S6 Kinase
RTKs	Receptor Tyrosine Kinases
SAPK	Stress-Activated Protein Kinases
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SFM	Serum-Free Media
SHR VSMCs	Vascular Smooth Muscle Cells Isolated from SHRs
SHRs	Spontaneously Hypertensive Rats
sIL-6R	Soluble Interleukin -6 Receptor
SOD	Superoxide Dismutase
STAT3	Signal Transducer and Activator of Transcription 3
TBST	Tween in Tris-Buffer Saline
TEMED	Tetramethylethylenediamine
TNF-α	Tumor Necrosis Factor-Alpha
TSC	Tuberous Sclerosis Complex
v/v	Volume by Volume
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VSMCs	Vascular Smooth Muscle Cells
Wistar VSMCs	Vascular Smooth Muscle Cells Isolated from Wistar Rats
ZIPK	Zipper-Interacting Protein Kinase
µL	Micro Liter
µM	Micro Molar

Chapter 1: Introduction

1.1. Background

At the dawn of the 19th century, Sir Henry Dale achieved a breakthrough that would herald a new era in endocrinology. He discovered that a chemical extracted from the human posterior pituitary gland caused uterine contractions in pregnant cats, dogs and guinea pigs. He called this chemical 'oxytocin,' derived from the Greek words '***ωχνξ***,' '***τοχοχξ***,' meaning 'swift birth.' Over the next 100 years, oxytocin became a subject for extensive research.[1]

It is now established that oxytocin is a neurohypophysial nonapeptide secreted by magnocellular cells in the paraventricular and supraoptic nuclei of the hypothalamus. It is stored in the posterior lobe of the pituitary gland. A wide range of stimuli including sucking (milk ejection reflex), pre- (labor induction) and post-parturition, and stress trigger oxytocin release from the posterior pituitary into the blood circulation. There is an extensive projection of oxytocinergic neurons in the central nervous system (CNS) — brain and spinal cord including amygdala, ventromedial hypothalamus, nucleus acumben, septum, and brainstem.[2] Besides, oxytocin is also synthesized peripherally in tissues such as placenta, uterus, testis, kidney, heart, and blood vessels in both vascular endothelial cells (ECs) and vascular smooth muscle cells (VSMCs).[3]

The oxytocin receptor gene (OXTRG) has been mapped in the human chromosome at the locus 3p25-3p26.2 alongside a gene that encodes the vasopressin receptor. The distance between the two genes is 500-kilo bases (kb). The human OXTRG spreading over 17 kb consists of three introns and four exons. The third intron, being the largest spanning 12 kb, separates the transmembrane domain 6 from the coding region. Exons 1 and 2 frame the 5' prime non-coding domain, while the exons 3 and 4 code for the amino acids sequence for oxytocin receptors. Exon 4, in particular, codes for the seven transmembrane domain, carboxyl terminal and the 3' non-coding region.[3]

Both oxytocin and vasopressin are the neurohypophysial peptides, with each having a sequence of nine amino acids, and are stored in the posterior lobe of the pituitary gland. The point of difference between the structure of oxytocin and vasopressin is the amino acid at position 8. In oxytocin, leucine is present at number 8. Whereas, in vasopressin, either lysine or arginine is present at this position. The sequence of amino acid in oxytocin is as follows: *Cysteine (1) – Tyrosine (2) – Phenylalanine (3) – Glutamine (4) – Asparagine (5) – Cysteine (6) – Proline (7) – Leucine (8) – Glycine (9)*. Importantly, the presence of isoleucine at position 3 in the oxytocin family is required for the activation of oxytocin receptors.[4]

Oxytocin receptors belong to the class I G-protein coupled receptors (GPCRs) that predominantly bind to $G_{q/\alpha 11}$ class of guanosine triphosphate (GTP) binding proteins that activate phospholipase C (PLC). This brings about the formation of the two important secondary messengers: inositol-3-phosphate (IP3) and 1, 2-diacylglycerol (DAG). IP3 sparks the release of intracellular calcium

(Ca^{2+}); DAG activates protein kinase C (PKC) that triggers the phosphorylation of targets leading to a further downstream signaling. The activation of these second messengers result in oxytocin-mediated physiological effects at different locations. When bound with G_i protein, oxytocin receptors inhibit the adenylyl cyclase (AC), leading to inhibition of the production of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). This inhibits cAMP pathway-dependent phosphorylation of targets and attenuates further downstream signaling. The effects of oxytocin varies widely and depends upon the target tissue(s), gland(s), organ(s) or system(s). [3,5,6]

1.2. Traditional roles of oxytocin

Oxytocin is often referred to as a 'female reproductive hormone.' It is known to contract pregnant uterus assisting parturition[1]. It also affects lactating breasts by initiating milk ejection reflex[7], promotes maternal-fetal bonding[8] and plays a role in reproduction and in reaching an orgasm.[9]

Oxytocin became commercially available in 1980. Its agonist and antagonists have found applications in both clinical research and pre-clinical studies.[10,11,12]

It has become common in medical practice to employ pharmacological interventions such as intravenous administration of Pitocin (a commercial brand name oxytocin), along with other mechanical methods (for example extra-amniotic Foley catheter) to induce labor.[13,14]

Oxytocin has emerged as a versatile hormone affecting social bonding, trust, memory, learning, empathy, and depression.[15,16,17,18]

From as recent as the year 2012, the intranasal administration of oxytocin has found an application in clinical behavioral studies.[19,20,21]

1.3. Emerging role of oxytocin in the cardiovascular system (CVS)

In the last decade, the dynamics in oxytocin research have been completely shifted. A new role for oxytocin has now evolved in cardiovascular regulation.[22,23] Oxytocin being considered initially as a female hormone is now gaining consensus as a 'cardiovascular hormone.'[24] The present study is an endeavor to develop a deeper understanding of the role that oxytocin plays in cardiovascular physiology and pathophysiological conditions.

1.3.1. Effect of oxytocin on heart

Several studies in rats and humans have reported that oxytocin is synthesized locally in the heart. The studies demonstrating competition binding curves have revealed only one receptor subtype for oxytocin in the heart. All the chambers of the heart and the large blood vessels have oxytocin and its receptors.[25,26]

The equilibrium dissociation constant (K_D) values are slightly different in rat heart (K_D ~ 1.00 nM), hypothalamus (K_D ~ 0.02 nM), and uterus (K_D ~ 0.76 nM). However, in the blood vessels there are no significant difference between the K_D values of rat vena cava (K_D ~ 0.78 nM) and aorta (K_D ~ 0.59 nM). The right atrium and the left ventricle have the highest and lowest concentrations of oxytocin, respectively. The right atrium's oxytocin levels are equivalent to hypothalamic oxytocin levels.[25]

Studies have shown that oxytocin can reduce the rate of cardiac blood flow and induce bradycardia. In isolated dog atriums, oxytocin produced negative inotropic and chronotropic effects via its receptors in the heart.[27,28]

1.3.2. Effect of oxytocin on cardiomyocytes

Oxytocin plays a role in the regulation of atrial natriuretic peptide (ANP).[29,30] The ANP is a peptide hormone consisting of 28-amino acids. It is synthesized, stored and released locally in cardiomyocytes. The factors that cause the secretion of ANP include atrial wall distension, increased blood volume (in pathological conditions such as congestive heart failure), angiotensin II (Ang II), endothelin, and sympathetic activation (norepinephrine). The ANP elicits a cardioprotective effect by decreasing the central venous pressure, thereby reducing blood volume and decreasing ventricular pre-load, and thus decreasing cardiac output. Besides, ANP also inhibits renin release and aldosterone synthesis.[31]

One previous study has demonstrated the functional relevance of oxytocin and its receptors in rat heart and cultured cardiomyocytes. The study has evidenced that oxytocin (10^{-6} M, a physiologically relevant concentration) stimulates ANP release from isolated perfused female rat hearts and cultured cardiomyocytes. In this study, not only had the oxytocin antagonist (10^{-6} M and 10^{-7} M) , in a concentration-dependent manner, inhibited the oxytocin-stimulated ANP release in the heart perfusion medium, but also inhibited the basal release.[29]

The research findings of these studies indicate that oxytocin, via ANP release, reduces cardiac contraction - force and rate, and decreases circulating blood volume. This illustrates the protective effect of oxytocin in cardiovascular physiology, and its benefits can be further explored in cardiovascular pathophysiological conditions, for instance, congestive heart failure.

In recent years, oxytocin has also found relevance in stem cell research on cardiogenesis. A few studies have indicated that oxytocin has the potential to induce differentiation of embryonic stem cells (P-19ECs) to cardiomyocytes (CMs). Studies have brought to light that nitric oxide (NO) signaling is involved in oxytocin-induced differentiation of P-19ECs to CMs.[32] There is also an upregulation of oxytocin receptors and Gata-4 (a protein required for the atria-ventricular formation and its functions) that are considered crucial to the oxytocin-mediated cardiomyocytes differentiation process.[33]

1.3.3. Effect of oxytocin on blood vessels (endothelial cells and VSMCs)

Several pre-clinical studies have demonstrated that oxytocin has shown anti-oxidant and anti-inflammatory effects in vascular endothelial cells (derived from the aorta), THP-1 monocyte, and macrophages. The physiological levels of oxytocin have shown significant reduction in nicotinamide adenine dinucleotide phosphate (NADPH) superoxide production, and also decreased the levels of the interleukin-6 (IL-6) in monocytes, macrophages, and aortic endothelial cells.[34]

It has been reported that in diabetes, an independent risk factor for many cardiovascular diseases (CVD),[35] neovascularization is significantly impaired after ischemia.[36] The endothelial cells lose the ability to recover significant blood flow due to endothelial dysfunction. Besides, the progenitor endothelial cells suffer reduced angiogenic functions.[37] Oxytocin has been shown to restore angiogenic functions in a streptozocin-induced diabetes rat model. The pre-treatment of cultured bone marrow-mesenchymal stem cells (MSCs) with oxytocin has shown significant restoration of kruppel-like factor 2 (KLF2) which is a crucial angiogenic factor.[38]

Many in vitro studies have shown that oxytocin, in endothelial cells derived from the human dermal microvasculature, umbilical vein and breast cancer cells, induces an overexpression of vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMP-11, MMP-17 and MMP-26), integrin β6 genes, and cathepsin D.[39,40] Thereby, stimulating migration and proliferation (which are the crucial steps in angiogenesis) of endothelial cells.

These studies have suggested that oxytocin induces angiogenic effects via an oxytocin receptor stimulation of the phosphatidylinositol-3-kinases (PI3K)/Akt/endothelial nitric oxide synthase (eNOS) pathway. It has been determined that the ability of oxytocin to phosphorylate Akt and eNOS depends on the PLC activity.[39] A subsequent study has revealed that it is through the stimulation of PLC and calcium mobilization, oxytocin phosphorylates the tyrosine kinases: proline-rich tyrosine kinase-2 (Pyk-2) and Src. This activates the PI3K/Akt/eNOS pathway.[41]

It is interesting to note that most of the cardiovascular effects of oxytocin have been studied in vascular endothelial cells and cardiomyocytes. However, in VSMCs, only the presence of oxytocin receptors has been established thus far. There is very little information regarding oxytocin effects in VSMCs.[42,43]

1.3.4. Effect of oxytocin in various pathophysiological cardiovascular conditions

Inflammation and oxidative stress are the two critical pathophysiological events in atherosclerosis.[44] In a rat model of atherosclerosis, the administration of oxytocin showed an improvement in the lipid profile and decreased the plasma levels of IL-6, monocyte chemoattractant protein-1 (MCP-1) and C reactive protein (CRP). It also improved the plasma levels of reduced glutathione (GSH) and nitric oxide (NO). Besides, oxytocin treatment significantly improved the histological structure of the aorta detected by a significantly decreased expression of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in a rat model of atherosclerosis.[45]

The ability of oxytocin to counteract oxidative stress and reduce inflammation underscores the importance of oxytocin in many other cardiovascular pathophysiological conditions including atherosclerosis.[45]

In myocardial infarction (MI), oxytocin has displayed a cardioprotective role. In a rabbit model of MI, oxytocin has been reported to protect against myocardial injury through the phosphorylation of kinases such as extracellular signal-regulated kinase (ERK), signal transducer and activator of transcription 3 (STAT3), Akt, MMP and eNOS. Akt, ERK, and STAT3 are all pro-survival signals that may play an important role in cardiac ischemic preconditioning, thereby reducing ischemic damage to the heart.[46]

In a rat model, oxytocin has mediated improvement in myocardial injury immediately after heart transplant by downregulating the chemotactic agents:

chemokine (C-X-C motif) ligand 1 (CXCL1), and macrophage inflammatory protein-2 alpha (MIP-2α)/Chemokine (C-X-C motif) ligand 2 (CXCL2). These agents are known to recruit neutrophils in the myocardium.[47] In ovariectomized rats, the oxytocin pre-treatment protected from injury after MI by reducing plasminogen activator inhibitor-1 (PAI-1) concentrations.[48]

1.4. Role of angiotensin II (Ang II) in the CVS and its pathophysiology

The emergence of the different functional aspects of oxytocin in the CVS continues to fascinate the scientific community. It is noteworthy that a central component of the CVS is the renin-angiotensin-aldosterone system (RAAS). The principal effector hormone of the RAAS is Ang II. It is an octapeptide, and an enzyme known as the angiotensin converting enzyme (ACE) catalyzes the conversion of angiotensin I (Ang I) into Ang II. Ang II affects all major organs of the body – heart, kidney, vasculature, and brain. In particular, Ang II has a diverse range of effects in the CVS.[49]

Ang II causes vasoconstriction and maintains the blood pressure in the normal physiological conditions.[50] Besides, it is also involved in many CVD such as atherosclerosis, congestive heart failure, diabetes, hypertension, inflammation and MI.[51] It mainly binds to the angiotensin type 1 receptors (AT-1R) in the CVS and execute its actions via stimulation of the mitogen-activated protein kinases (MAPKs) pathways, PI3K/Akt/mammalian target of rapamycin (mTOR) pathways, and Rho kinase pathways.[51],[52] These signaling pathways and kinases are involved in Ang II-mediated contraction of VSMCs, hypertrophy, hyperplasia, endothelial dysfunction, vascular diseases, and their progression.[53,54]

In pathological conditions such as hypertension and atherosclerosis, Ang II significantly impacts the repair and the remodeling of VSMCs in large blood vessels. It phosphorylates ERK1/2 and p38 MAPKs, thereby inducing expression of immediate early response genes: c-fos, c-myc and c-jun. Thus, Ang II mediates cell proliferation, migration, and hypertrophy in VSMCs.[55,56] Besides, Ang II

causes the migration of VSMCs on receiving pathogenic stimuli by inducing STAT3 phosphorylation, and its subsequent nuclear translocation induces expression of the genes specific to cell proliferation, hypertrophy, and migration of VSMCs.[57,58]

Ang II is also a known pro-oxidant.[59] It causes production of reactive oxygen species (ROS) via the activation of NADPH oxidase, leading to increased oxidative stress. The Ang II-induced oxidative stress causes endothelial dysfunction and vascular inflammation by the activation of NF-kB, a redox-sensitive nuclear transcription factor, and upregulation of adhesion molecules: intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), selectin, integrin, cytokines [IL-6, IL-12, IL-1, tumor necrosis factor-alpha (TNF-α)], and chemokines (MCP-1, IL-8, osteopontin).[60] It has been reported that glutathione s-transferase (GST) attenuates Ang II-induced STAT3 phosphorylation and exerts a protective effect in VSMCs. GST conjugates the reduced form of glutathione (a cellular antioxidant) with reactive oxygen species (ROS) and detoxifies it.[58]

Ang II has been shown to influence fibrinolysis activity.[51,61] A key regulator of endogenous fibrinolytic activity and thrombolysis resistance is PAI-1. It physiologically inhibits plasminogen activation. In response to vascular injury, PAI inhibits cellular migration and matrix degradation.[62,63] Ang II infusion in normotensive and hypertensive patients showed increased circulating levels of PAI-1.[64] In a rat model, Ang II via interactions with AT1R, increased the expression of PAI in VSMCs from isolated aorta, thereby influencing endogenous fibrinolytic

activity.[65,66] PAI-1 is an important cardiovascular risk factor in atherosclerosis. Besides, an increased PAI-1 also manifests amongst the metabolic abnormalities associated with hypertension.[67,68]

The antagonizing of Ang II effects by employing ACE inhibitors is a well-established therapy commonly used in CVD. The ACE inhibitors are in clinical practice in the management of hypertension, coronary artery disease, heart failure, diabetes, and chronic kidney diseases.[69] They are one of the few treatment options that we have currently for these diseases. However, there are many side effects attributed to ACE inhibitors' use in CVD.[70] Despite the long clinical use of these agents, treatment of hypertension has not been all that successful. The need of the present times is to identify alternative targets and unveil better therapeutic approaches to better treat CVD.

1.5. The gap in the literature, research hypotheses, and study aims

As mentioned earlier in the background, oxytocin has demonstrated cardioprotective effects in various in-vitro and in-vivo studies performed on vascular endothelial cells and cardiomyocytes.[23] However, there is a gap in the literature. Even though previous researches have established that oxytocin receptors are found in VSMCs, interestingly, oxytocin effects in VSMCs have not been fully characterized. On the contrary, the effects of Ang II, another crucial cardiovascular peptide, have been well established in VSMCs and the CVS to a significant extent. As stated before, Ang II is known to induce vascular oxidative stress, phosphorylate ERKs, PI3Ks, and Rho kinases, leading to marked VSMCs proliferation, hypertrophy, hyper-contraction, inflammation, and increased oxidative stress.[51] Therefore, to fill in the gap regarding the lack of research on the effect of oxytocin on VSMCs and its interaction with Ang II, the present study was conducted.

In the context of the protective effects of oxytocin in the cardiovascular system and from the results of the preliminary experiments, the following research hypotheses were proposed:

1.5.1. Hypothesis 1

By its ability to reduce proliferation, inflammation, oxidative stress, and vasocontraction pathway, oxytocin antagonizes Ang II-induced proliferation, inflammation, and oxidative stress and dysregulates Ang II-induced vasocontraction pathway in primary Wistar aortic VSMCs.

1.5.2. Hypothesis 2

Oxytocin signaling pathways in aortic VSMCs are altered in hypertensive conditions.

The hypotheses mentioned above were tested by pursuing the following specific aims:

1.5.3. Specific Aim 1

To characterize the effect of oxytocin on proliferation, inflammation, oxidative stress and vasocontraction in primary aortic VSMCs obtained from normotensive Wistar rats. The VSMCs proliferation was determined by employing cell proliferation, cell viability, and cell growth assays. To further understand the mechanism of how oxytocin reduces aortic VSMC proliferation, the two critical signaling pathways involved in VSMCs proliferation were investigated: ERK1/2 pathway and PI3K/Akt pathway. The oxidative stress was measured as the function of the ROS levels, and inflammation was assessed by measuring IL-6 levels. The effect of oxytocin on vasocontraction was assessed indirectly by investigating oxytocin's effect on the Rho-kinase pathway [Rho-associated coiled-coil kinase-1 (ROCK-1) and -2 (ROCK-2)] involved in the vasocontraction of VSMCs.

1.5.4. Specific Aim 2

To determine of the interaction between oxytocin and Ang II in primary aortic VSMCs obtained from normotensive Wistar rats. The interaction of oxytocin with Ang II was investigated by studying the effect of pretreatment of oxytocin on Ang II-induced cell proliferation and proliferation

pathways (the ERK1/2 pathway and PI3K/Akt pathway), inflammation, oxidative stress, and the Rho-kinase pathway in aortic VSMCs obtained from normotensive Wistar rats.

1.5.5. Specific Aim 3

To characterize the effect of oxytocin on proliferation, inflammation, oxidative stress and vasocontraction in primary aortic VSMCs obtained from spontaneously hypertensive rats (SHRs). The effects of oxytocin on VSMC proliferation (ERK1/2, PI3K, Akt), oxidative stress (ROS), inflammation (IL-6) and vasocontraction (ROCK-1 and ROCK-2) were characterized on VSMCs isolated from SHRs. The comparison was made, and the analysis was carried out between the effects of oxytocin on its own and its pre-treatment with Ang II in aortic VSMCs Wistar rats and SHRs.

1.6. Role of VSMCs in the CVS and its pathophysiology

VSMCs constitute tunica media, the middle layer of blood vessels, with endothelial cells and connective tissues making tunica intima and tunica externa, respectively (**Figure 1**). VSMCs are the stromal cells which perform a variety of functions and play a crucial role in the CVS.[71]

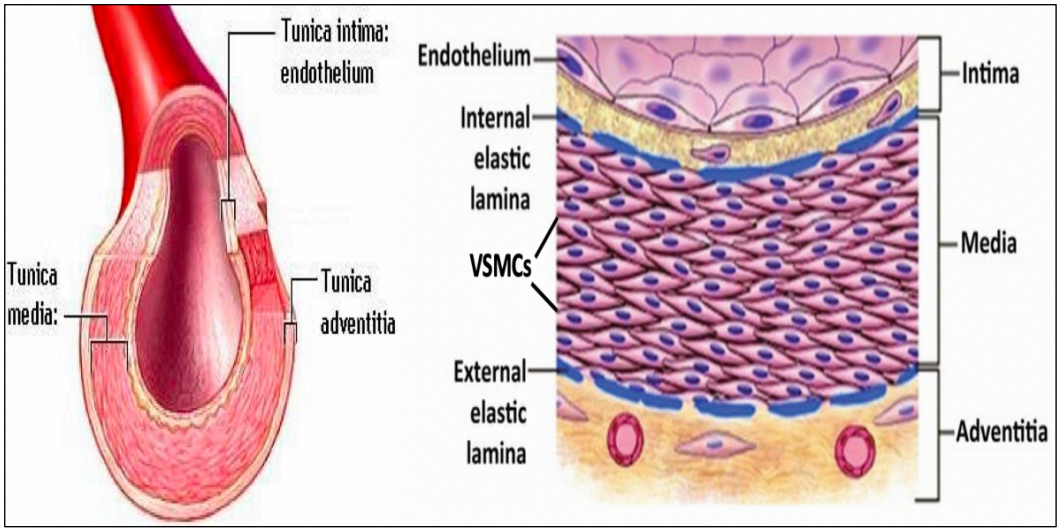

Figure 1. Histology of a blood vessel. *VSMCs form the middle layer (tunica media) of a blood vessel. Adapted from Muscle cell and Tissue, Intech Open. 2018;230.*

VSMCs maintain the contractile tone of the arterial wall via intracellular calcium release causing the myosin-actin interactions, regulating blood pressure and flow to meet the metabolic demands of the body. They secrete molecules that comprise the extracellular matrix (ECM) which enable arteries to withstand the high pressure of the circulating blood.[72]

In normal physiological conditions, VSMCs remain quiescent and adhere to their phenotype essential in regulating contractile tone of arteries. Molecules such as laminin, perlecan, heparan sulfate proteoglycan (HSPG), and collagen type

IV, composing the basement of VSMCs are attributed to keeping VSMCs quiescent.[73]

However, in the event(s) of pathological or pathophysiological condition(s) such as arterial injury, an excessive overload of lipids, pressure-related stress, hyperglycemic conditions, VSMCs undergo a variety of adaptations and remodeling. They become active and transition into mitogen-responsive phenotypes in the presence of fibronectin.[74] They start interacting with the intracellular signaling pathways, involving the ERKs, PI3Ks, and Rho kinases.[75,76]

The activation of these pathways leads to hypercontraction, proliferation and migration of VSMCs often leading to the thickening of the tunica intima, hyperplasia, precipitating aneurysm, hemorrhage, and restenosis, and worsening the other cardiovascular diseases including atherosclerosis, hypertension and increasing the complications post-vascular surgical procedures. [72,77]

The activated phenotype of VSMCs also produce ROS which play a role in the vascular pathology through its redox potential.[78], The principal enzymatic source of the production of ROS in VSMCs is NOX. In hypertension, vasoactive agents such as Ang II activate the NOX, leading to the increased production of ROS, ultimately resulting in increased oxidative stress and increased expression of inflammatory mediators such as cytokines (example IL-6).[79]

The functions of VSMCs in physiology and their role in the pathophysiology of the CVS are crucial and render them a potential target of therapeutic approaches in cardiovascular research. Anti-proliferative therapies

such as rapamycin and paclitaxel targeting VSMCs' hyperplasia in animal models have failed to show similar effects in patients.[80]

Therefore, there is a need to develop therapies that are not only targeting the most notable pathophysiological features of CVD such as proliferation, migration, hypercontraction, oxidative stress, and inflammation of VSMCs but are also maintaining contractile phenotype of VSMCs.

1.7. Study significance

It was envisioned that this study would be a step towards the long-term goal of identifying oxytocin as an alternative therapeutic target in the treatment of CVD. The present study was an attempt to determine the potential role of oxytocin with regards to its cardioprotective effects against Ang II-induced deleterious effects. Previous studies have established that at sustained high levels, Ang II acts as the 'aggressor' in the cardiovascular system.[53] On the contrary, pre-clinical evidence have, so far, indicated a 'protective' role of oxytocin in the cardiovascular system.[23]

This in vitro study was the first of its kind to investigate and understand the protective effects of oxytocin against the harmful actions of Ang II, with relation to VSMCs, on the cardiovascular system. The study findings contributed towards a better understanding of oxytocin actions on blood vessels (VSMCs), and identification of secondary messenger pathways involved in this oxytocin effect.

The research findings demonstrated that oxytocin blocked Ang II-mediated hyperproliferation of VSMCs and proliferation and vasocontraction pathways in VSMCs from SHRs, thus providing evidence of oxytocin's vital role in cardiovascular pathophysiology and it shows the potential therapeutic benefits it might have in the management of hypertension. The study generated significant pre-clinical evidence regarding the beneficial effects of oxytocin in a hypertensive rat model, laying a platform foundation for future mechanistic in vivo studies and pilot clinical studies.

1.8. Barriers, issues, limitations, and delimitations

The present study involved the use of primary rat aortic VSMCs. The advantage of using laboratory-cultured primary VSMCs is that they retain many cellular functions and markers quite similar to the ones observed in vivo. Whereas, commercially available VSMCs cell lines may differ phenotypically and genetically from their origin. However, there were specific challenges that occurred during the course of this study.

Firstly, the procurement of significant yield of high purity VSMCs was challenging. The techniques utilized in this research to harvest primary cells was the 'explant method.'[81] Although this technique is cost-effective and routinely applied to obtain a high yield of VSMCs, its major drawback is the purity of primary cells obtained. That VSMCs are prone to natural contamination with intimal endothelial or adventitial fibroblasts, the VSMCs isolation protocol was optimized, and the cells were used from passages between 3-15 for maintaining the phenotypical uniformity and the purity of cells.[81] The endothelial cells and fibroblast eventually die out by the time VSMCs underwent the third passage.

Secondly, the effect of many cell passages was a challenge. For avoiding this, care was taken to make sure that all the experiments in both Wistar rats and SHRs were conducted using the same passage number. For example, if a cell proliferation experiment was carried out with VSMCs in the passage 4 in Wistar rats, the VSMCs from the similar passage number was utilized to perform a cell proliferation experiment in SHRs.

Lastly, the magnitude of the response of cells to the treatments obtained from one rat was different from the other rat. Therefore, to reduce the variation in data and increase the reproducibility of the data, the experiments were repeated numerous times using multiple rats to increase the number of replicates. However, the repetition of all the experiments was tough, time-consuming and challenging.

Chapter 2: Literature Review

2.1. MAPK/ERK1/2 pathway in the CVS

MAPKs belong to the serine-threonine specific class of kinases. They constitute a signal transduction pathway known as the MAPK pathway. The MAPK pathway is a cascade that converts signals from external mechanical stimuli and growth factors into signal transduction, leading to the regulation of genes involved primarily in protein synthesis.[82] It occurs in sequential phosphorylation of the three core protein kinases from cytosol: mitogen-activated protein kinase kinase kinase (MAPKKK), mitogen-activated protein kinase kinase (MAPKK), and mitogen-activated protein kinase (MAPK).[83] A schematic representation of the activation of MAPK signaling pathway is shown in the **Figure 2.1**.[84]

It is the MAPKs which carry out intracellular signal transduction through three distinct pathways. The first one is through the phosphorylation and activation of extracellular signal-regulated kinase (ERK), which has two isoforms: ERK1 and ERK2. The phosphorylated ERK1 and 2 (active form) lead to the phosphorylation and activation of various regulatory proteins in the cytosol as well as in the nucleus, which is critical for protein synthesis and cell survival.[83]

The second MAPK pathway involves the phosphorylation and activation of stress-activated protein kinases (SAPK). This cascade of MAPK

is triggered in response to external stimuli such as ultraviolet rays, stress (heat shock), altered osmolarity, hypoxia, and exposure to reactive oxygen species.[85]

The triggering of the second MAPK pathway causes the phosphorylation and activation of c-Jun N-terminal kinases (JNK) which is critical in proliferation, apoptosis, inflammation, and in response to deoxyribonucleic acid (DNA) damage.[86–88]

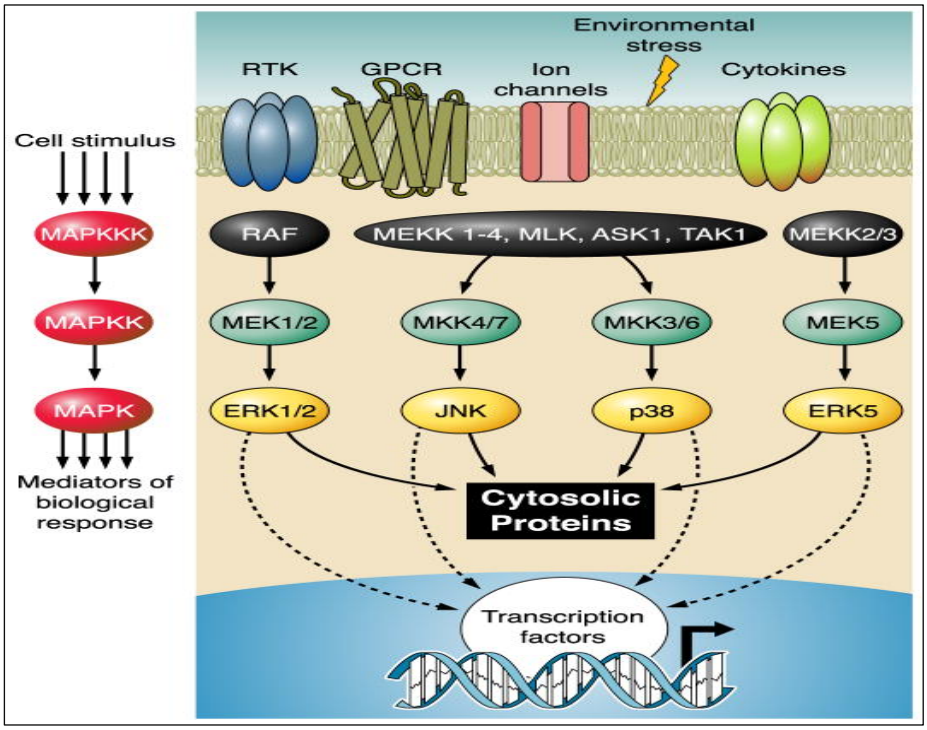

Figure 2.1. Activation of mitogen-activated protein kinase (MAPK) signaling pathway in the cardiovascular system. *Adapted from Physiol Rev. 2010;90(4);1508. GPCR indicates G-protein coupled receptor; RTK, receptor tyrosine kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; RAF, rapidly accelerated fibrosarcoma ; MLK, mixed-lineage kinases; ASK-1, apoptosis signal-regulating kinase 1; TAK-1, transforming growth factor-β-activated kinase 1; MAPKKK/MEKK, mitogen-activated protein kinase kinase kinase; MAPKK/MEK/MKK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase.*

The third pathway consists of p38 MAPK. Like the JNK pathway, this pathway can also be triggered in response to stress induced by a change in cellular osmolarity, exposure to free radicals, heat shock, and inflammation.[89] The p38 MAPK signaling is involved in cellular processes such as proliferation, differentiation, migration and immune response.[90–92]

The ERK1/2 pathway is one of the first MAPK pathways to be characterized in mammals. In VSMCs, the stimulation of AT1R by Ang II, causes the phosphorylation of ERK1/2. The phosphorylated ERK1/2 (active form) then translocate to the nucleus, and subsequently result in the phosphorylation of transcription factors including c-Fos, c-Jun, and activator protein-1 transcription factors, ultimately carrying out cell synthesis, proliferation, differentiation and migration of VSMCs. In addition, ERK1/2 targets the ribosomal S6 kinase (RSK). Once phosphorylated, the activated ERK1/2, in turn, phosphorylates RSK via the transfer of ribonucleic acid (RNA)-binding factor. This is a key step in the initiation of translation, leading to protein synthesis, regulation, and progression of the cell cycle of VSMCs.[93–95]

Phosphorylated ERK1/2 is also known to be involved in the regulation of vascular tone of VSMCs. It has been established that h-Caldesmon (h-CD) is a downstream target of the phosphorylated ERK1/2.[96] An h-CD is a high molecular weight contractile regulatory protein involved in the contractile responses of vascular walls.[97]

Numerous studies have implicated ERK1/2 signaling in the pathophysiology of CVDs such as hypertension.[98,99] In an in vivo study,

increased activity of ERK1/2 was reported after transient elevation of blood pressure with Ang II in femoral arteries and rat aortas.[100]

The pharmacological inhibition of ERK1/2 signaling was shown to improve endothelial function, and significantly reduced Ang II-induced contraction of mesenteric resistance arteries in spontaneously hypertensive rats (SHRs).[100] In transgenic mice models, ERK1/2 signaling was shown to stimulate hypertrophy in cardiomyocytes via phosphorylation of factors such as elk-1, necessary for the activation of the c-Fos gene.[101]

Oxytocin was demonstrated to exert differential effects on the ERK1/2 signaling pathway. For example, oxytocin-induced phosphorylation of ERK1/2 increased the proliferation of cardiomyocytes and vascular endothelial cells.[40,102] In other instances, such as in human embryonic kidney (HEK293) cells, oxytocin exhibited proliferative as well as anti-proliferative actions. The dual action of oxytocin on the proliferation of HEK293 cells was determined by the location of the oxytocin receptors. Oxytocin reduced the phosphorylation of ERK1/2, and as a result, inhibited cell proliferation when the oxytocin receptors were present outside the caveolin-1-enriched microdomains. And, oxytocin augmented phosphorylation of ERK1/2, resulting in stimulated cell proliferation when the oxytocin receptors were inside caveolin-1-enriched microdomains.[103]

It is imperative to understand the role of caveolins. Caveolins are scaffolding proteins involved in the regulation of GPCR signaling. They facilitate GPCR regulation via scaffolding and partitioning of different receptors, G-proteins trafficking, and signaling.[104]

As stated earlier, oxytocin's effect on cell proliferation and ERK1/2 activity has been studied in cardiomyocytes and vascular endothelial cells. However, little to no information is available on the effects of oxytocin on ERK1/2 signaling pathway and on its effects on the proliferation of VSMCs.

The present study characterized the effect of oxytocin and its impact on Ang II (known for stimulating cell proliferation via the ERK1/2 signaling[55]) on cell proliferation in aortic VSMCs from normotensive rats (Wistar rats). Further, oxytocin's impact on aortic VSMCs proliferation and on ERK1/2 signaling, and oxytocin's effect on Ang II-mediated cell proliferation and Ang II-stimulated ERK1/2 signaling were also investigated in aortic VSMCs from SHRs.

2.2. PI3K/Akt signaling in the CVS

The phosphoinositide-3-kinase (PI3K)/Akt pathway is an essential intracellular signaling transduction pathway involved in the regulation of the CVS.[105] It is crucial in the proliferation of VSMCs,[106] control of the vascular myogenic tone, and inflammation.[107,108] Over the last decade, the PI3K/Akt signaling has been implicated in various pathological conditions of the CVS [109] such as cardiac hypertrophy (a critical risk factor of heart failure),[110] and hypertension. [107]

Phosphoinositide-3-kinases (PI3Ks) are intracellular enzymes which catalyze the phosphorylation of phosphatidylinositol (PI). PI are lipids (phosphatidyl glycerides).[111] PI3Ks phosphorylate PI at the 3-hydroxyl group of the inositol ring. Based on the products of the catalysis, PI3Ks have been grouped into three classes: Class I PI3Ks, which form phosphatidylinositol-3,4-biphosphate (PI-3,4-P_2); Class II PI3Ks, which form phosphatidylinositol-3-phosphate (PI-3-P), PI-3,4-P_2, and phosphatidylinositol triphosphate (PIP_3); and Class III PI3Ks, which generate PI-3-P. All classes of PI3Ks are present in cardiomyocytes, vascular endothelial cells, and VSMCs.[112]

A general structure of a PI3K includes three domains: C-2 domain, helical domain, and a catalytic domain. The C-2 domain binds to the membranes, the helical domain serves regulatory functions, and the catalytic domain carries out the kinase activity of PI3Ks. Based on the regulatory domain, the Class I PI3Ks are further subdivided into two categories: Class I A PI3Ks which bind to receptor tyrosine kinases (RTKs), and Class I B PI3Ks which bind to GPCRs. The Class I

A PI3Ks exist as heterodimers of different isoforms of α, β, and δ of the p110 catalytic domain. Whereas, the Class I B PI3Ks exist as heterodimers of p110γ and p101 catalytic domains.[113] All Class I PI3Ks are significant in the CVS and its pathophysiology.[114] Their actions are antagonized by phosphatase and tensin homolog deleted on chromosome ten (PTEN).[115]

Akt is a serine-threonine kinase and also known as protein kinase B (PKB). Akt is a downstream signaling target of PI3Ks. There are three isoforms of Akt: Akt 1 (PKBα), Akt 2 (PKBβ), Akt 3 (PKBγ). All forms of Akt are present throughout blood vessels, heart, lungs, and brain.[116] The stimulation of GPCRs by peptides such as Ang II or growth factors results in the activation of PI3Ks. The activated PI3Ks lead to the formation of PI-3,4-P_2 and PIP_3, which in turn phosphorylate Akt (active form).[117]

The phosphorylated form of Akt triggers downstream signaling via the phosphorylation of downstream signaling molecules such as mTOR. mTOR is a serine-threonine kinase. The phosphorylation of mTOR by phosphorylated Akt is vital to protein synthesis by the PI3K/Akt signaling pathway. mTOR has two isoforms: mTORC1 and mTORC2. The phosphorylated mTORC1 stimulates cell proliferation via the phosphorylation of p70S6 kinase, eukaryotic initiation factor 4E (elF4E), and 4E-binding protein 1 (4E-BP1). The activated p70S6 kinase phosphorylates and activates S6 ribosomal protein. The activated elF4E acts as translator initiator. It phosphorylates 4E-BP1. A phosphorylated 4E-BP1 is required for protein translation. The phosphorylated mTORC2 stimulates the

phosphorylation of Akt and is involved in the autoregulation of mTOR.[118] The PI3K/Akt signaling pathway is illustrated in the following figure (**Figure 2.2**):

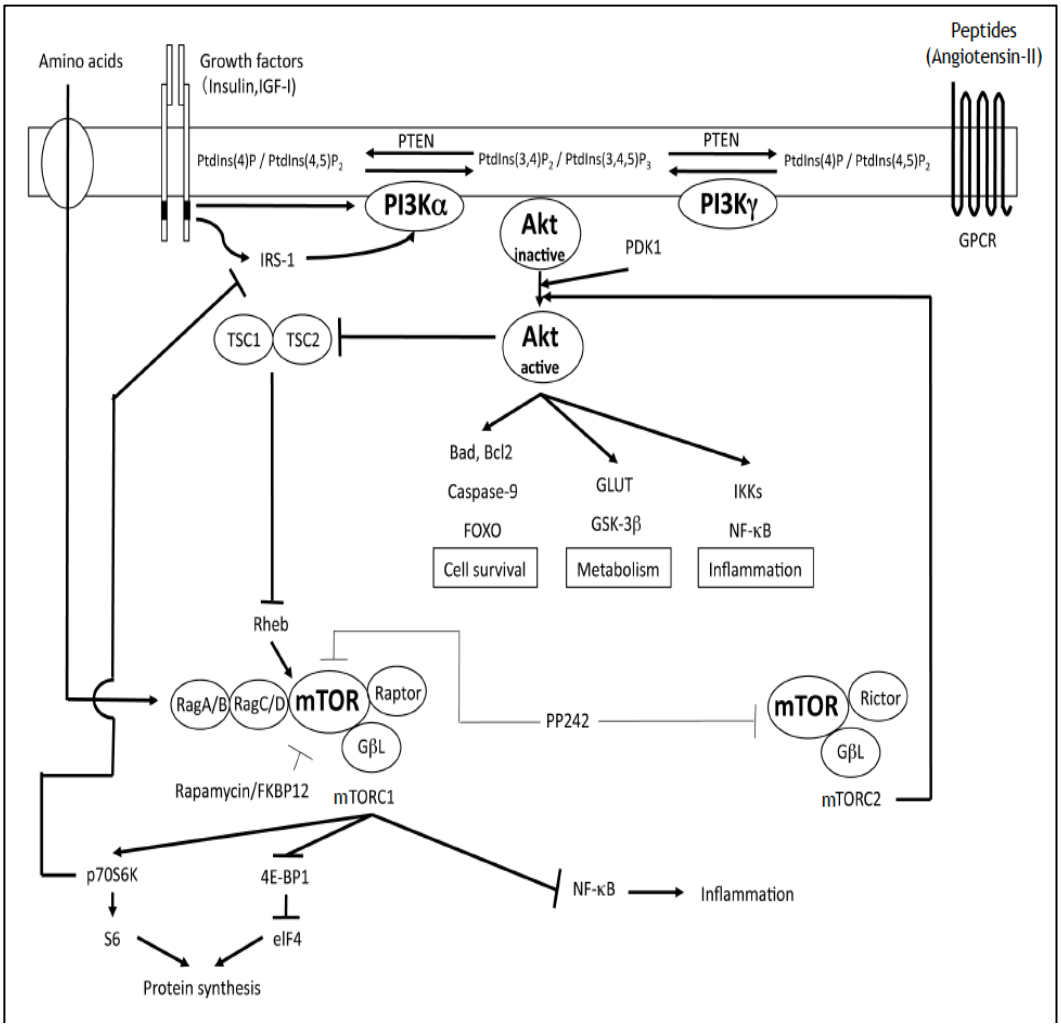

Figure 2.2. The PI3K/Akt signaling pathway in the cardiovascular system. *A schematic representation of the PI3K/Akt signaling pathway in cardiomyocytes, vascular endothelial cells, and vascular smooth muscle cells (VSMCs). Adapted from Curr Pharm Des. 2011;17(18):1819. FKBP indicates FK506-binding protein; GLUT, glucose transporter; GPCR, G-protein coupled receptor; GSK, glycogen synthase kinase; IGF, insulin-like growth factor; PDK, phosphoinositide-dependent kinase; PI3K, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; PTEN, phosphatase and tensin homolog deleted on chromosome ten; p70S6K, p70S6 kinase; TSC, tuberous sclerosis complex; eIF4, eukaryotic initiation factor 4.*

The implication of PI3K/Akt signaling cascade in many cardiovascular pathophysiological conditions has been reported by previous studies.[119] In hypertension, it has been shown that the PI3K/Akt pathway alters vascular function mainly by increasing the myogenic tone of resistance arteries and by enhancing the constricting activity of VSMCs. A myogenic tone can be described as the functional behavior of resistance arteries in response to perfusion pressure.[107] On the activation of GPCRs by cardiovascular peptides such as Ang II, the PI3K/Akt signaling results in an increased intracellular level of calcium via the L-type voltage-gated calcium channel (L-VGCC).[120,121]

It is noteworthy that L-VGCC dysfunction is an important characteristic feature of CVDs such as hypertension and cardiac arrhythmia, and its targeting by the compounds known as calcium channel blockers has been a long-standing clinical practice in the management of hypertension and arrhythmia.[122,123]

Several studies have documented the relevance of PI3Ks in hypertension.[107,124,125] A study conducted using a transgenic mice model revealed that the chronic administration of Ang II did not produce hypertension in mice lacking PI3Kγ. The lack of PI3Kγ not only reduced the L-VGCC-mediated intracellular calcium reflux but also decreased VSMCs contractility when stimulated with Ang II.[121]

Another relevant aspect of the PI3K/Akt signaling in the physiology and pathophysiology of the CVS is its role in cardiac hypertrophy.[110] It is pertinent to highlight that cardiac hypertrophy can be both beneficial and harmful. Beneficial

cardiac hypertrophy is called ‘physiological cardiac hypertrophy,’ whereas the harmful hypertrophy is known as ‘pathological cardiac hypertrophy.’[126]

Physiological cardiac hypertrophy is usually seen and is associated with natural post-natal development, pregnancy, and exercise. Thus, this is an adaptive growth of cardiomyocytes. The characteristic features of physiological cardiac hypertrophy are the preservation of contractile functions of the sarcomere (the contractile unit of cardiomyocytes) and a lack of interstitial fibrosis.[127,128] On the other hand, pathological cardiac hypertrophy is ‘maladaptive hypertrophy.’ It is characterized by increased cell volume of cardiomyocytes, the new formation of sarcomeres, interstitial fibrosis and inflammation.[129]

Several studies have demonstrated PI3K/Akt-mediated cell signaling in both physiological and pathological cardiac hypertrophy.[130,131] In a study employing a transgenic mice model, mice with dominant negative PI3K showed a significant reduction in exercise-induced physiological cardiac hypertrophy compared to wild type mice (control).[132] In another study employing a similar transgenic mice model, mice deficient in PI3K showed protection against isoproterenol-induced pathological cardiac hypertrophy and thus protection from heart failure.[133]

It is thought-provoking that oxytocin is being touted as a ‘cardioprotective’ hormone,[23] yet it has not been investigated for its effects on PI3K/Akt signaling in cardiomyocytes, vascular endothelial cells, and VSMCs. To partially address this gap in the scientific literature, the present study used an in vitro model to investigate

the effects of oxytocin alone and its pre-treatment with Ang II on the PI3K/Akt signaling pathway in VSMCs in both normotensive and hypertensive conditions.

2.3. Rho kinases (ROCK-1 and ROCK-2) signaling in the CVS

Rho-associated coiled-coil kinases (ROCK) -1 and -2 belong to the serine/threonine category of kinases. They are the targets of small GTPases (that bind and hydrolyze the guanosine triphosphates) : Rho A, Rho B, and Rho C.[134] These GTPases are found abundantly in VSMCs and are rapidly activated by vasoconstricting peptides such as Ang II.[135] Both ROCK-1 and ROCK-2 are involved in the organization and remodeling of actin cytoskeleton, extracellular matrix, and in the contraction of VSMCs.[136,137] They are also critical in cell adhesion, motility, and proliferation.[138,139] Pre-clinical research and clinical evidence have implicated ROCK signaling in the pathogenesis of various diseases of the CVS including angina pectoris, hypertension, pulmonary arterial hypertension, heart failure, and stroke.[140–144]

The structural features of ROCKs include phosphorylation and cleavage sites. The structure of both ROCK-1 and ROCK-2 has an N-terminal containing a kinase domain, coiled-coil region, Rho-binding domain (RBD), and a C-terminal comprising of a PH-domain containing a cysteine-rich C1 domain. An RBD possesses three zones known as a ‘switch’: Switch I, Switch II, and Switch III, with each having a strong binding affinity for Rho A, Rho B, and Rho C, respectively. The C1-domain of C-terminal is involved in the autoinhibition of ROCKs. The two isoforms of ROCK have the highest homology in their kinase domain (about 92 percent) and the least homology in their coiled-coil domain (about 55 percent).[145,146]

The activation of ROCKs depends on the catalytic activity of both the N-terminal and C-terminal regions. The ROCKs form homodimers, and dimerization depends upon kinase domains, an extension of N-terminals, and coiled-coil regions. Following an interaction of RBD with guanosine-5'-triphosphate (GTP)-loaded Rho proteins (Rho-A, -B, -C), the phosphorylation of the hydrophobic motif near the C-terminal region occurs, causing the kinase domain to undergo conformational changes from a closed (inactive) to an open (active) state.[147,148] The active state of ROCKs is essential in carrying out the downstream cell signaling in the following three different ways:

First, the activated form of ROCKs directly augments the phosphorylation of myosin phosphatase target subunit-1 (MYPT-1). Second, it stimulates the phosphorylation of zipper-interacting protein kinase (ZIPK) which in turn phosphorylates MYPT-1.[149,150] Third, activated ROCKs phosphorylate C-kinases potentiated protein phosphatase 1 inhibitor -17 (CPI-17). A CPI-17 is a direct inhibitor of MYPT-1.[151,152]

The phosphorylation of MYPT-1 prevents the binding of myosin light chain phosphatases (MLCP) with myosin light chain (MLC), thus boosting myosin light chain kinase (MLCK)-induced phosphorylation of actin leading to sustained contraction of VSMCs. The stimulation of the ROCK signaling results in decreased activation of MLCP, and increased contraction of VSMCs which ultimately leads to elevated blood pressure.[151,152] The ROCK-mediated contraction of VSMCs is depicted in the **Figure 2.3**.

In animal models of hypertension, an increased ROCK signaling has been observed which is further enhanced by the intake of a high-salt diet.[153] An increased ROCK signaling in hypertension has been attributed to the dysregulation of Rho guanine nucleotide exchange factors (Rho-GEFs).[154,155] In clinical studies, an elevated phosphorylation level of MYPT-1 has been reported in patients diagnosed with essential hypertension. Ang II-induced hypertension and cardiac hypertrophy have also been linked with hyper-ROCKs signaling.[156,157]

In samples of heart-tissues obtained from patients with heart failure, truncated ROCKs have been found. A cleavage of C-terminal of ROCKs by the activation of pro-apoptotic enzymes such as caspases abolishes the autoinhibitory capability of ROCKs. This leads to constitutive activation of ROCKs, resulting in a sustained hypercontraction of blood vessels, fibrotic cardiomyopathy, and diastolic dysfunction.[158]

These detrimental effects of truncated ROCKs were further supported by findings from a study carried out on cardiomyocytes from mice. The cardiomyocytes showed an increased expression of C-terminally nicked ROCK-1, and the histopathological findings presented the characteristics of fibrotic cardiomyopathy.[159]

Over the years many compounds have been developed to target the ROCK signaling. A wide range of those compounds are often non-selective ROCK inhibitors (i.e., they are non-specific in their binding to the two ROCK isoforms). They compete for ATP in the kinase domain of ROCKs, thus acting as competitive

inhibitors.[160] Fasudil is the only ROCK inhibitor that has been approved for clinical use for the treatment of various CVD including hypertension. [161]

Medical interventions employing fasudil in patients with hypertension, pulmonary arterial hypertension (PAH), angina pectoris, and heart failure have shown significant improvement in their medical conditions.[142,162–164] The administration of fasudil in patients with congenital heart defects significantly reduced pulmonary artery pressure and pulmonary vascular resistance.[165]

Evidence presenting an increased expression of ROCK-2 in the pulmonary arteries and pulmonary VSMCs in patients with idiopathic PAH has demonstrated the role of ROCK signaling in PAH.[166] This was further strengthened by another study in which patients with PAH treated with fasudil resulted in acute pulmonary vasodilation.[167] Chronic treatment with fasudil not only resulted in an improvement in pulmonary hypertension, but also reduced right ventricular hypertrophy, and prevented further remodeling in the pulmonary vasculature, along with significant decrease in VSMCs proliferation and hypercontraction, and lower endothelial dysfunction.[168]

Previous studies have highlighted enhanced activity of ROCK in patients with acute and chronic heart failure.[169] ROCK activity measured in terms of phosphorylated levels of total MYPT-1 in circulating leukocytes indicates the involvement of ROCK signaling in heart failure.[170] Also, stimulated ROCK activity has been found in patients with myocardial infarction. A study in which fasudil was administered to patients with heart failure showed a significant decrease in vascular resistance in the forearm and a noticeable increase in forearm vasodilation.[171]

Another clinical study showed the beneficial effect of ROCK inhibitors in patients with angina. The treatment with fasudil resulted in reduced number of angina attacks per week in patients with stable angina and also increased their maximum exercise time.[162,164]

The literature presented above underlines a crucial role of ROCK signaling pathway in physiological and pathophysiological conditions of CVS. Interestingly, oxytocin effect on ROCKs' signaling has not been previously studied. The present study endeavored to investigate the effect oxytocin, and its impact on Ang II-stimulated ROCK signaling in aortic VSMCs isolated from normotensive and hypertensive rats.

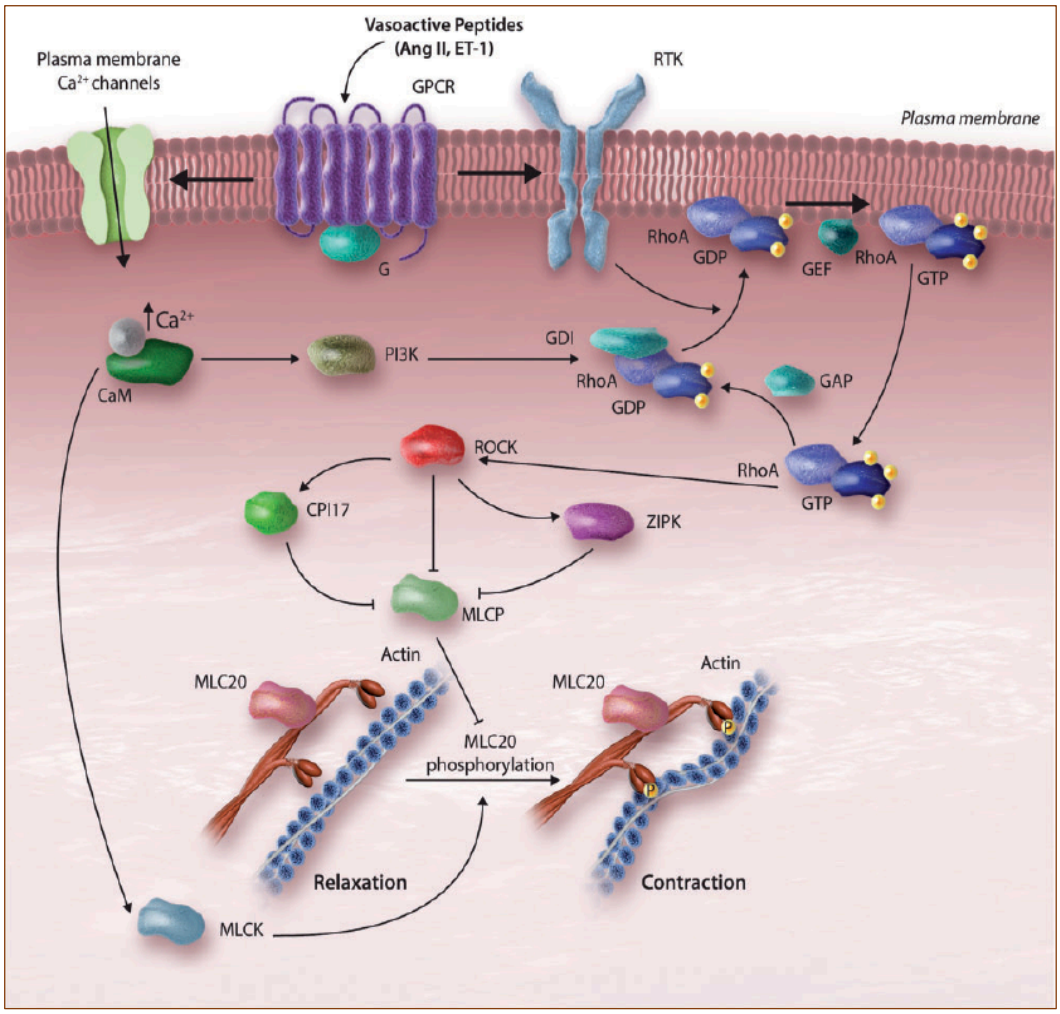

Figure 2.3. ROCK signaling pathway in the contraction of VSMCs. *Adapted from Cardiovasc Res. 2018;114(4):534. Ang II indicates angiotensin II; ET-1, endothelin-1; GPCR, G-protein coupled receptor; RTK, receptor tyrosine kinase; GDP, guanosine diphosphate; GTP, guanosine triphosphate; GEF; guanine nucleotide exchange factor; GDI, guanine nucleotide dissociation inhibitor; PI3K, phosphoinositide-3-kinase; ROCK, rho-associated coiled-coil kinase; CPI-17, C-kinases potentiated protein phosphatase 1 inhibitor-17; MLCP, myosin light chain phosphatases; ZIPK, zipper-interacting protein kinase; MLCK, myosin light chain kinase.*

2.4. Interleukin-6 in the CVS

IL-6 is a cytokine which has pleiotropic effects.[172] It is crucial in the CVS and its diseases.[173] IL-6 is involved in the process of inflammation eliciting a myriad of immunological responses such as activation of thymocytes, stimulation and activation of T-cells and B-cell differentiation, differentiation and galvanization of macrophages, activation of natural killer (NK) cells, and stimulation of hepatocytes for the production of inflammatory proteins such as the CRP.[174–181]

IL-6 binds to the IL-6 receptor complex to induce the signal transduction. The IL-6 receptor complex is comprised of two membrane-bound glycoproteins: IL-6R and glycoprotein 130 (gp130). The IL-6R is an 80 Kilo dalton (KD) component which binds to a ligand. The gp130 is the signal transducing part of the IL-6 receptor complex with an atomic mass of 130 KD.[182] Also, there is a soluble form of IL-6R (termed sIL-6R). The binding of IL-6 to the membrane-bound IL-6R has been attributed to normal physiological immune responses of IL-6.[183]

However, the binding of IL-6 to the soluble form (IL-6/sIL-6R) has been implicated in various pathological conditions,[184,185] and has been clinically targeted by anti-IL-6 agents such as tocilizumab in severe cases of rheumatoid arthritis (RA).[186,187,188,] The IL-6 and its receptors both soluble and membrane-bound are present ubiquitously in all tissues, and abundantly in endothelial cells, VSMCs, and cardiomyocytes in particular.[189–191]

The effects of IL-6 are both protective and detrimental.[192] The duration of pathogenic stimuli or conditions determines these actions whether they would be

beneficial or deleterious.[193,194] Short-term exposure to pathogenic stimuli results in protective effects of IL-6, whereas, a long-term exposure culminates in IL-6 driven harmful effects. A classic example of such consequences is the effects of IL-6 on the heart. IL-6 signaling in cardiomyocytes is cardioprotective in response to a short-term pathophysiological condition.[194–196] However, when that pathophysiological condition remains for a long time, then the IL-6 signaling induces pathological cardiac hypertrophy, reduces cardiac contractility, ultimately leading to decreased cardiac function.[193,197,198] Studies have highlighted IL-6 mediated decreased cardiac contractility is associated with the JAK/STAT pathway and not the ERK pathway.[199]

IL-6 has been implicated in the pathogenesis of coronary artery disease such as atherosclerosis.[200] Previous studies have shown the IL-6-mediated upregulation of MMPs contribute to vascular remodeling and atherosclerotic plaque disruption.[201] Many studies have demonstrated higher serum levels of IL-6 in patients diagnosed with and hospitalized for unstable angina.[202]

The increased expression of myocardial IL-6 has been associated with the progression of heart disease.[203,204–206] Studies have established elevated levels of IL-6 in patients with heart failure. More specifically, the concentrations of IL-6 were directly related to the severity of left ventricular dysfunction and to the extent of the activation of the sympathetic system and RAAS.[207]

Ang II, an effector hormone of the RAAS, is known to induce inflammation in VSMCs via stimulation of IL-6 signaling.[208] Oxytocin, on the other hand, has shown anti-inflammatory actions via decreasing IL-6 signaling in vascular

endothelial cells.[34] The effects of oxytocin on inflammation in VSMCs have not been studied. Interestingly, its interaction with Ang II with regards to inflammation has also not been investigated. This study undertook the investigation of oxytocin's effect on inflammation, and its interaction with Ang II in aortic VSMCs isolated from both normotensive rats and hypertensive rats.

2.5. Reactive oxygen species (ROS) in the CVS

The ability of a living organism to convert oxygen into water through aerobic phosphorylation forms the core of the metabolism and production of energy. This very process of the metabolism of oxygen yields a byproduct known as 'ROS.'[209] These toxic species are extremely deleterious in nature. They cause severe damage to cellular DNA, RNA, lipids, and proteins, resulting in cellular apoptosis, necrosis, and inflammation.[210–212] ROS play a significant role in the development of various pathological condition of the CVS including hypertension, cardiac hypertrophy, heart failure, diabetes, and atherosclerosis.[78,213–216]

The primary source of ROS in cells in general, and in vascular endothelial cells, VSMCs, and cardiomyocytes, in particular, is an enzyme called 'nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.'[217]

The catalytic component of NADPH oxidase is NOX. There are seven isoforms of NOX that have been identified, and five of them are found in human: NOX1, NOX2, NOX3, NOX4, and NOX5.[79,218]

In cardiovascular cells, the generation of ROS is stimulated by neurohumoral vasoconstrictor peptides such as Ang II and Endothelin -1 (ET-1), and by norepinephrine (NE). It is well known that activation of AT-1R by Ang II, triggers the production of superoxide (O_2^-) by NADPH oxidase. Studies have demonstrated the production of O_2^-, by NADPH oxidase, resulting in vasopressor action in response to Ang II infusion in normotensive conditions (Wistar rats).[219,220] The reaction of NADPH oxidase-mediated production of highly reactive superoxide is as follows:

$$2O_2 + NADPH \rightarrow 2 \cdot O_2^- + NAD(P)^+ + H^+$$

ET-1 is also known to induce ROS production via the activation of NADPH oxidase. ET-1 is a potent vasoconstrictor of the endothelium layer of blood vessels. There are two isoforms of ET receptors: ET_A and ET_B. The stimulation of ET_A receptors induces ROS production via NADPH oxidase activation and results in vasoconstriction of endothelium. The ET_B receptors have the opposite effect. They mediate vasorelaxation of endothelium.[221]

ROS production in VSMCs and cardiomyocytes is also induced through the stimulation of alpha-1 adrenergic receptors (α1-ADR) by NE.[222] The AT-1R, ET-1, and α1-ADR activate NADPH oxidase via interaction with G-proteins and thus generate ROS.[223]

The other sources of ROS production are mitochondrial transport chain and eNOS uncoupling. In addition to ROS production by NADPH oxidase, ROS generated via NOS uncoupling and in mitochondria contribute to pathogenesis of the CVDs such as hypertension.[224–227]

Under normal physiological conditions, the production of NO occurs via the coupling of L-arginine, in the presence of tetrahydrobiopterin (BH_4) acting as a co-factor, with eNOS. On stimulation by pathogenic stimuli, the levels of BH_4 are reduced, leading to the uncoupling of eNOS. eNOS uncoupling results in the release of O_2^- from the oxygenase domain of eNOS. The increased production of O_2^- causes endothelial dysfunction and eventually precipitates vascular damage.[224,228,229]

The dysfunction of the mitochondrial respiratory chain due to vascular damage under pathophysiological conditions causes the transfer of electrons to molecular oxygen and bring about the formation of ROS such as O_2^- and hydrogen peroxide (H_2O_2).[230–232] Increased mitochondrial ROS production damages mitochondrial antioxidants such as manganese superoxide dismutase (MnSOD), in turn, further initiating ROS production.[233] The imbalance between ROS and antioxidants creates oxidative stress which is at the core of the pathogenesis of many CVD including hypertension.[234] Redox signaling in the CVS is elucidated in the following figure:

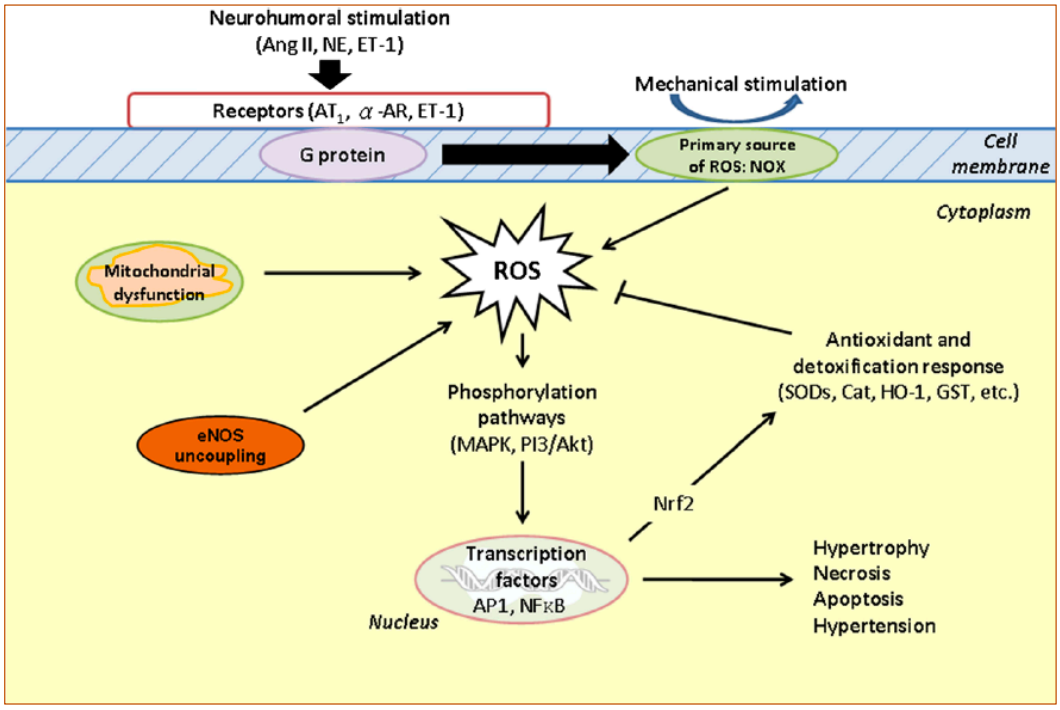

Figure 2.4. Redox signaling in the cardiovascular system. *Adapted from J Biomed Sci.2013;20(69):4. AT-1 indicates angiotensin-1 receptors; α-AR, alpha-adrenergic receptors; ET-1, endothelin-1; Ang II, angiotensin II; NE, norepinephrine; ROS, reactive oxygen species; HO-1, heme oxygenase-1; Cat, catalase; AP-1, activator protein-1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor (erythroid-derived 2)-like 2;*

MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3-kinase; eNOS, endothelial nitric oxide synthase.

Oxidative stress is known to induce activation of MAPKs (ERK1/2, JNK, and p-38 MAPK).[235,236] Many studies have demonstrated that the induction of oxidative stress under pathophysiological conditions increases ROS production, which in turn activates various MAPKs, leading to activation of several transcription factors, for example, activator protein-1 (AP-1), NF-κB, and nuclear factor (erythroid-derived 2)-like 2 (Nrf2). These transcription factors are involved in hypertrophy of cardiomyocytes, endothelial cells and VSMCs, necrosis, apoptosis, and inflammation which are characteristic features of many CVD.[237,238]

Previous research has shed light on the effect of oxytocin on oxidative stress in cardiomyocytes and vascular endothelial cells. However, the impact of oxytocin on oxidative stress in VSMCs has not been investigated thus far. The present study examined oxytocin's effect on oxidative stress in VSMCs via ROS measurement. Also, the interaction of oxytocin with Ang II, a known pro-oxidant, was studied in aortic VSMCs isolated from normotensive rats and hypertensive rats.

2.6. Summary

The present study was conducted to address the gap in the literature regarding oxytocin's effect on proliferation, proliferation pathways (ERK1/s and PI3K/Akt signaling pathways), and vasocontraction pathways (ROCK pathway) in VSMCs. The study also evaluated the impact of oxytocin on oxidative address (assessed by measuring ROS levels) and inflammation (analyzed by measuring IL-6 levels). To characterize these effects of oxytocin on VSMCs, this study employed aortic VSMCs isolated from Wistar rats (normotensive) and SHRs (hypertensive rats).

Chapter 3: Methodology

3.1. Wistar rats and SHRs

The present study employed aortic VSMCs isolated from Wistar rats and SHRs. Hypertension has been extensively studied pre-clinically in SHRs models, often using normotensive Wistar rats as control.[239,240] The SHR is a well-established genetic animal model of primary hypertension. The major advantage of the SHR model is the lack of inter-individual variation. Similar to the progression of hypertension in humans, SHRs have pre-hypertensive, developing and sustained hypertensive phases.[241,239]

3.2. Isolation and culture of primary rat aortic VSMCs

The study protocol was approved by the Nova Southeastern University Institutional Animal Care and Use Committee (NSU-IACUC). Adult male Wistar rats (300-350g) and spontaneously hypertensive rats [(SHRs) 300-350g] were euthanized in a carbon dioxide (CO_2) chamber as per the established guidelines. VSMCs were isolated and cultured as per the previously established explant method. [242]

The thoracic cavity was cut-open, the aorta was isolated, and placed in a sterile 100 mm Petri dish containing sterile phosphate-buffered saline (PBS). Sterile forceps were utilized to clean the aorta by removing adjacent fat layers and coagulated blood.

Once cleaned, the aorta was slit from top to bottom and cut into two pieces using sterile scissors. Each piece was then cut into a 1 mm strip and each strip was placed in a sterile 100 mm Petri dish containing Dulbecco's Modification of Eagle's Medium (DMEM) [(Corning Cellgro, NY)], supplemented with 10% (v/v) fetal bovine serum (FBS) glutamine (2 mM/L), streptomycin (100 1g/mL) and penicillin (VWR Life Science, GA). Petri dishes containing strips of freshly isolated aortic VSMCs were incubated at 37°C under standard humidified conditions (95% air and 5% CO_2) to allow VSMCs to grow out from strips onto the surface of petri-dish. Once VSMCs were outgrown, strips were removed, and VSMCs were allowed to grow in petri-dish. On reaching confluency, VSMCs were passaged in T-75 flasks so that enough number of cells were obtained to be able to conduct experiments using 100-mm Petri dishes, 6-well plates, 24-well plates, and 96-well plates.

Before every experiment, VSMCs were subjected to trypsinization (Corning™ 0.25% Trypsin, Fischer Scientific, FL), counted using the Hemocytometer method, and were seeded in either 100 mm Petri dishes, 6-, 24-, or 96-well plates depending on the experimental design. They were incubated at 37°C under previously described standard humidified conditions. After every 48 hours, the culture medium was replenished until VSMCs were about 90% confluent. Once confluent, VSMCs were washed with sterile PBS. VSMCs were made quiescent by replenishing with the serum-free media (SFM) for 24 hours before commencing with the experimental treatments. Cell passages from 3-15 were used for all experiments. All experiments were performed using the primary aortic VSMCs isolated from at least three rats or more. [81]

3.3. VSMCs proliferation assessment by the Hemocytometer method

3.3.1. The principle of the Hemocytometer method

The Hemocytometer method is the most widely used technique used to determine the number of cells per unit volume of a suspension.[243] It is a valuable research tool utilized to count cells in cell proliferation studies. It consists of a microscopic slide which is divided into two chambers. Each chamber has grid lines which make nine large squares. Each large square has an area of 1mm². The large squares are demarcated from each other by compact grid lines. The volume of the entire counting grid lines is 0.9 mm^3 in each chamber. A glass cover-slip is placed over a chamber following which a suspension of cells is introduced into the chamber, and the cells are counted under the microscope. The cells are excluded from counting if they are touching the right-side and the bottom (**Figure 3.1**).

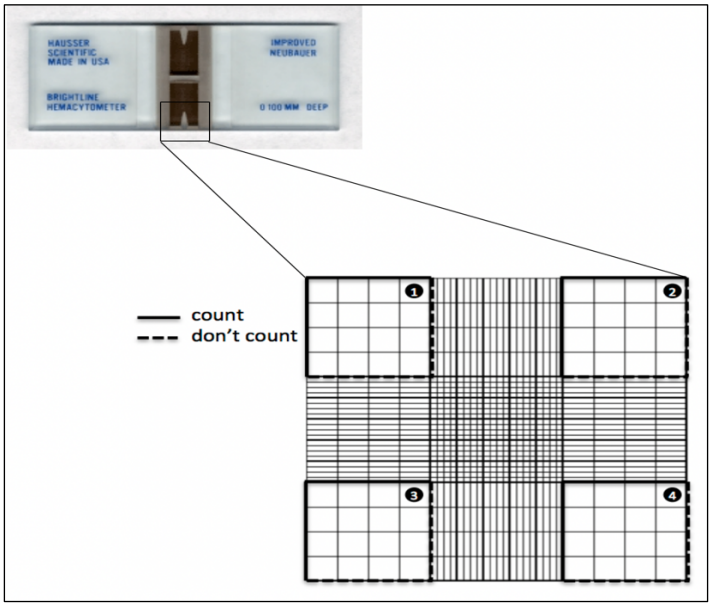

Figure 3.1. Hemocytometer and the grid lines of a chamber in a hemocytometer microscopic slide. *Adapted from https://www.hemocytometer.org/wp-content/uploads/2013/06/hemocytometer-squares-to-count.png?w=551.*

The hemocytometer technique employs the staining of cells with dyes such as trypan blue to identify live and dead cells. The cytoplasm of dead cells readily absorbs trypan blue and turns the appearance of dead cells to blue, making it easy to distinguish from live cells which have intact cytoplasm that does not take up trypan blue.[243]

3.3.2. The procedure of the cell counting by the hemocytometer method

Aortic VSMCs were seeded at 1×10^6 cells/well in 6-well plates and cultured as mentioned before. The cells were made quiescent by replacing serum-media with SFM, 24 hours prior to treatment. Post-treatment, the cells in each well were subjected to trypsinization and centrifuged at 1,500 rpm at 21°C for 10 minutes. The pellet was used to make the cell suspension with 1000 µL SFM, of which 100 µL was pipetted out and transferred in an Eppendorf tube, and stained with 900 µL 0.4% trypan blue. From the trypan blue-stained cell suspension, 10 µL was applied to the hemocytometer (Bright-Line Hemocytometer Set, Hausser Scientific, VWR Life Science, GA).

The cover-slip was placed on top to allow the complete and gentle filling of both chambers of the hemocytometer. The grid lines of the hemocytometer were focused under a microscope with a 10X objective. The cells were counted in all the 4 sets of large squares in the four corners of a chamber of the hemocytometer. The cells were excluded from counting when present on the right-hand boundary line or touching the bottom. The stained cells (dead cells), and unstained cells (live cells) were calculated and the dilution factor was corrected as per the manufacturer's protocol.[243,244]

3.4. VSMCs viability determination by the MTT assay

3.4.1. The principle of the MTT assay

The MTT assay measures cell viability as a function of the mitochondrial activity. It is a colorimetric assay which relies on the reduction of the yellow colored 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial enzymes such NADPH oxidase and mitochondrial succinate, into an insoluble purple colored formazan. Organic solvents such as isopropanol and dimethyl sulfoxide (DMSO) are employed to solubilize the insoluble formazan, and the color change is measured as 'optical density' by a microplate reader. The optical density is a function of the mitochondrial activity. The higher optical density is indicative of higher mitochondrial activity, and the higher mitochondrial activity relates to the higher number of metabolically active cells.[245]

3.4.2. The procedure of the MTT assay

Aortic VSMCs were seeded at 1×10^4 cells/well in 96-well plates. The serum-media was replaced with SFM 24 hours prior to treatment. After carrying out the treatments with different groups, the SFM-containing the different treatments was aspirated, and 50 µL of MTT (ACROS Organics, NJ) was added to each 96-well. The 96-well plates were incubated at 37°C under standard humidified conditions for 45 minutes. Following the incubation, 100 µL of DMSO (Merc KGaA, Germany) was added to each well. The plates were read at 570 nm using a Biotek microplate reader (Biotek-Synergy H1 Hybrid Reader, VT).[244]

3.5. VSMCs growth measurement by the ^{3}H-Thymidine incorporation assay

3.5.1. The principle of the ^{3}H-Thymidine incorporation assay

The tritiated-Thymidine (^{3}H-Thymidine) incorporation assay is considered as the gold-standard method to assess cell proliferation. This assay involves the direct measurement of cell proliferation by determining the synthesis of DNA. It employs the incorporation of the radioactive nucleoside tritiated (^{3}H)-Thymidine into strands of chromosomal DNA of the actively dividing cells, during the mitotic phase of cell division. The radioactivity recovered from the cells is measured using a liquid scintillation counter as 'counts per minute.' The cell growth is assessed as the function counts per minute (cpm).

A higher radioactivity count reflects higher cell growth. The cells undergoing active division resulting in higher cell growth will have more incorporation of ^{3}H-Thymidine into their DNA strands and consequently will have higher radioactivity count than those that are undergoing lesser cell division. Hence, a higher reading of cpm reflects a higher cell growth, and a lower reading of cpm represents a lower cell growth.[246]

3.5.2. The procedure of ^{3}H-Thymidine incorporation assay

Aortic VSMCs were seeded at 5×10^{4} cells/well in 24-well plates, cultured and made quiescent, as described previously, 24 hours before starting the treatments. Quiescent cells were subjected to different treatments. After the first 24 hours of treatment, 5 µL of ^{3}H-thymidine (1 µCi/mL) [Thymidine (Methyl-^{3}H), MP Biomedicals, LLC, CA], was added to each well. The plates were incubated at 37°C under standard humidified conditions for another 24 hours. In the end, the SFM

containing the treatments and ^{3}H-thymidine was aspirated and washed with sterile cold-PBS (1,000 µL), and 500 µL of cold 10% trichloroacetic acid (TCA) was added to each well (this was done to precipitate out remnants of any radioactive material present outside cells). The 24-well plates were incubated overnight at 4°C.

TCA was removed and 500 µL of 0.1 N sodium hydroxide (NaOH) – 2% sodium dodecyl sulfate [(SDS), NaOH-SDS] solution, previously maintained at room temperature (25°C), was added to each well and incubated for one hour at 4°C. This step was carried out to cause alkaline lysis of cells resulting in protein denaturation (breaking of double-stranded DNA into a single-stranded DNA). SDS solubilizes the cell membrane, and NaOH disrupts the hydrogen bonding between the DNA bases and thus causing protein denaturation).

The samples were collected in vials (20 mL). To each sample, 6 mL of liquid scintillation cocktail (Ultima Gold™, PerkinElmer Inc., CA) was added, and the samples were quantified by a PerkinElmer liquid scintillation counter (Tri-Carb® 2810TR Liquid Scintillation Analyzer, PerkinElmer Inc., CA) as per the manufacturer's protocol.[247]

3.6. VSMCs apoptosis and necrosis assessment by flow cytometery

3.6.1. The principle of flow cytometery

Flow cytometry is a technique commonly used in determining the characteristics of a single cell in suspension. It involves the measurement of the optical and fluorescence properties of cells including their nuclei and chromosomes. The technique relies on the use of fluorescent dyes, for example Annexin V and propidium iodide, that are specific in their binding to cellular components such as membrane phospholipids, DNA, and RNA. When cells in a suspension labeled with fluorescent dyes are passed through a light (laser) source, they rise to a higher energy state. When cells return to their resting state, they emit light energy at higher wavelengths. Multiple fluorochromes with similar excitation and different emission wavelengths enable the simultaneous determination of several properties of cells.[248]

This technique is also valuable in cell sorting and is widely utilized in clinical hematology. When cells continue to move straight in the direction of the light source, the process is called ‘forward-scatter,’ and when they deviate from the direction of the light source, it is referred to as ‘side-scatter.’ The larger cells demonstrate forward-scatter whereas, the smaller cells show side-scatter.[249] A flow diagram of a typical flow cytometer is shown in **Figure 3.2**.

Flow cytometry has profound applications in cell viability studies. It is used in the detection of apoptosis and necrosis.[250] Apoptosis is defined as ‘programmed cell death’ whereas necrosis is 'premature cell death' due to external factors including injury and acute infection.[251] Apoptosis is characterized by specific

morphological changes such as loss of plasma membrane asymmetry, membrane blebbing, shrinking of cytoplasm and nucleus, and DNA cleavage in the nucleosome. One of the earliest features of a cell undergoing apoptosis is the loss of plasma membrane asymmetry. It is characterized by the translocation of phosphatidylserine (PS), a membrane phospholipid, from the inside to the outside of the plasma membrane (**Figure 3.3**).[252]

A fluorochrome-labeled dye such as Annexin V binds to the PS of plasma membranes of an apoptotic cell and is detected by flow cytometer. Similarly, a fluorescent 7-amino actinomycin D dye (7-AAD) binds to broken fragments of DNA, the hallmark of necrosis, and thus enable the detection of cells undergoing necrosis by flow cytometer.[253]

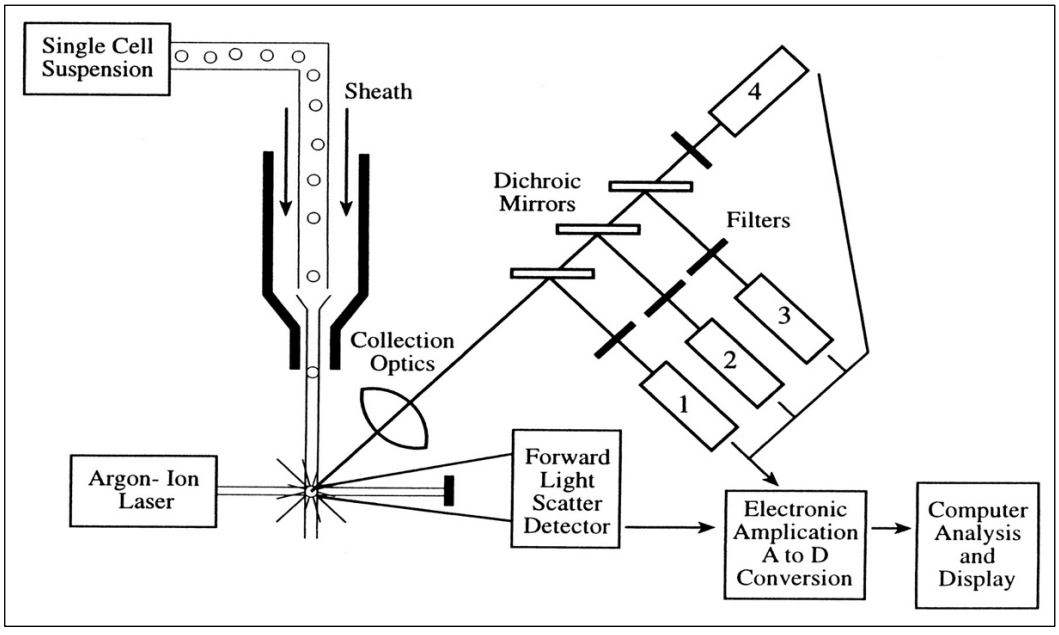

Figure* 3.2. *Flow cytometer. *A flow diagram of a typical flow cytometer analyzing a single cell suspension. The sample containing a single suspension is projected with a sheath fluid. The argon-ion laser intersects the cells and causes the forward-scatter and the side-scatter. The signals are detected, amplified, registered, and digitally analyzed in a computer. Adapted from Clin Chem. 2000;46(8 Pt 2):1222.*

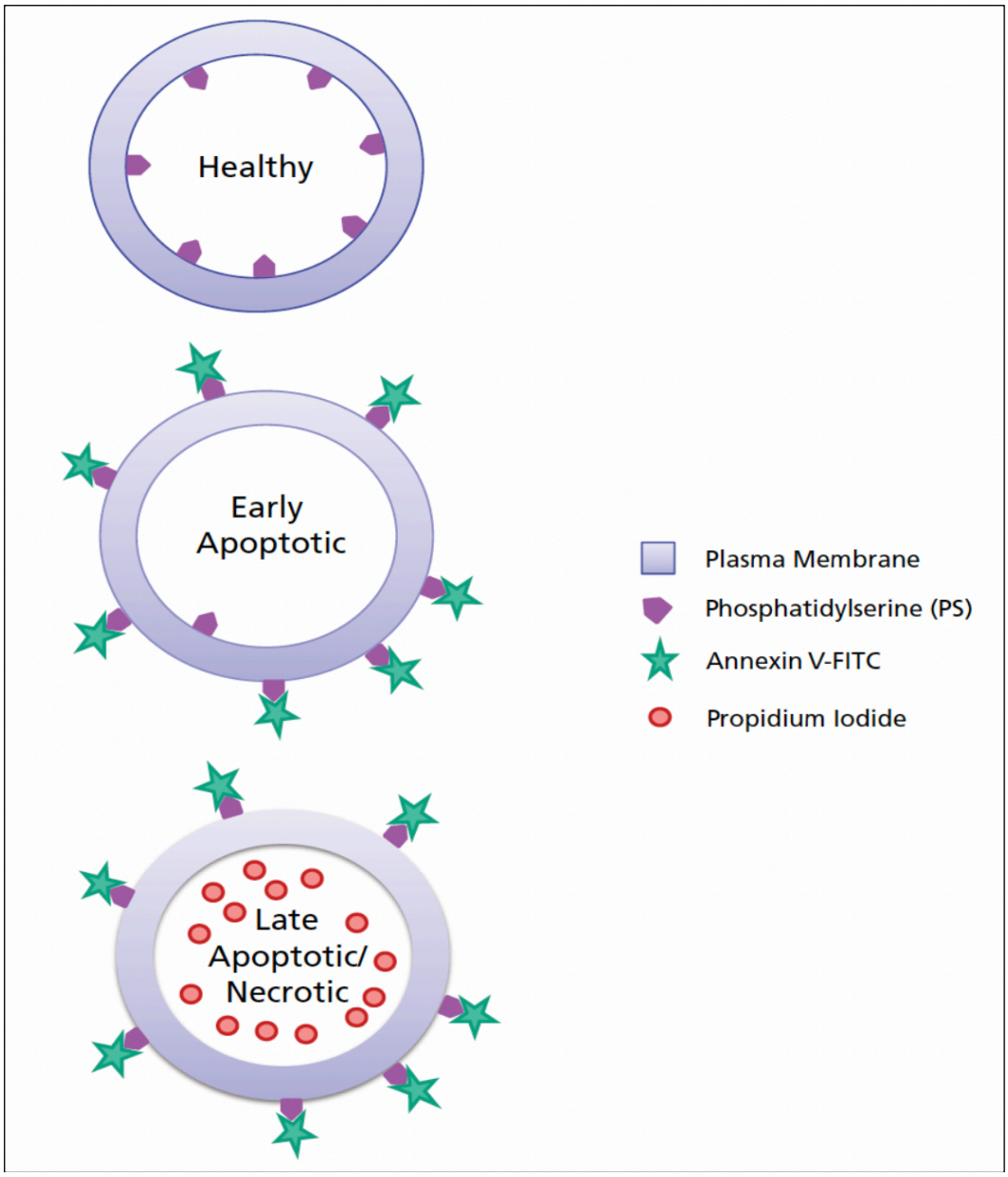

Figure 3.3. Presentation of markers for detection by healthy, apoptotic, late-apoptotic and necrotic cells. *Adapted from BD Biosci. 2011; 8:2.*

3.6.2. The procedure of apoptosis and necrosis assessment using flow cytometer

The present study utilized flow cytometry to study the effect of oxytocin on cell viability, apoptosis, and necrosis in aortic VSMCs from Wistar rats and SHRs. Aortic VSMCs were made quiescent 24 hours before the treatments. On the day of the assay after replenishing with the serum-free media (SFM), the treatment was performed, and cells were placed under standard incubation conditions for 24 hours. Post-treatment, the supernatant was removed. The cells were washed with 1,000 µL of sterilized PBS (previously maintained at the room temperature). The PBS was then removed, and 400 µL of Accutase cell detachment solution (# A6964 Sigma-Aldrich®, MO) was added to each well. The cells were then incubated under standard incubation conditions for about 5 -7 minutes.

Following the incubation, 1,000 µL of fluorescent-activated cell sorting (FACS) buffer (# MB-086-0500 Rockland™ antibody & assays) was added to each well, and the samples were collected in Eppendorf tubes (1.5 mL). The samples were then subjected to centrifugation at 1,500 rpm at 21°C for 10 minutes. The supernatant was discarded via decantation (the vacuum was not used to remove the supernatant in an effort to prevent cell loss). The pellet was re-suspended with the cold PBS (1,000 µL), and samples were re-centrifuged at 10,000 rpm for 5 minutes.

The supernatant was discarded, and the pellet was re-suspended with 100 µL Annexin V binding buffer and vortexed. To each prepared sample, 4 µL of Annexin V and 4 µL of 7-AAD (# 640922, FITC Annexin V Apoptosis Detection

Kit with 7-AAD, BioLegend®, CA) was added, and gently mixed and allowed to stand for 30 minutes.

Subsequently, 400 µL of binding buffer was added to each sample, and the samples were run at the medium speed (with the limit set at 10,000 events) using the BD LSRFortessa™ X-20 cell analyzer as per the manufacturer's protocol.[254]

The data generated represents four sections: percentage of live cells; percentage of cells positive for Annexin V; percentage of cells positive for 7-AAD; and percentage of cells positive for both Annexin V and 7-AAD. The cells positive for Annexin V indicate the percentage of cells undergoing apoptosis. The cells positive for 7-AAD reflect the percentage of cells undergoing necrosis. And, the cells that were double positive for both Annexin-V and 7-AAD represent the cells undergoing both apoptosis and necrosis.

3.7. IL-6 measurement by the enzyme-linked immunosorbent assay (ELISA)

3.7.1. The principle of IL-6 ELISA

ELISA is an immunological assay used for the detection and quantification of peptides, antibodies, cytokines, and hormones in biological samples. The general principle of ELISA is based on the interaction of antigen present in the biological sample with the specific antibody immobilized to a solid surface, and then its interaction with a secondary antibody conjugated with an enzyme. The detection is carried out by measuring the activity of the enzyme-linked by incubating it with a substrate, yielding a quantifiable product.[255]

The present study employed ELISA to measure IL-6 levels secreted by VSMCs in the culture media. For this purpose, rat IL-6 ELISA kits were purchased (Rat IL-6 Immunoassay, # R6000B Quantikine® ELISA, R&D Inc., MN). The package comprised of microplates previously coated with monoclonal antibodies for rat IL-6. The samples were loaded into the microplates. A secondary antibody (specific to rat Il-6) was then added making a sandwich of immobilized antibody specific for rat IL-6, samples (containing IL-6 antigen), and secondary antibody.

The secondary antibody conjugated with horseradish peroxidase (HRP) produced a colored or fluorescent reaction when a chemical substrate was added. The fluorescent signals were detected by a Biotek microplate reader (Biotek-Synergy H1 Hybrid Reader, VT), thus determining the presence and the quantity of IL-6 antigen in the samples. A typical ELISA sandwich is depicted in the following figure:

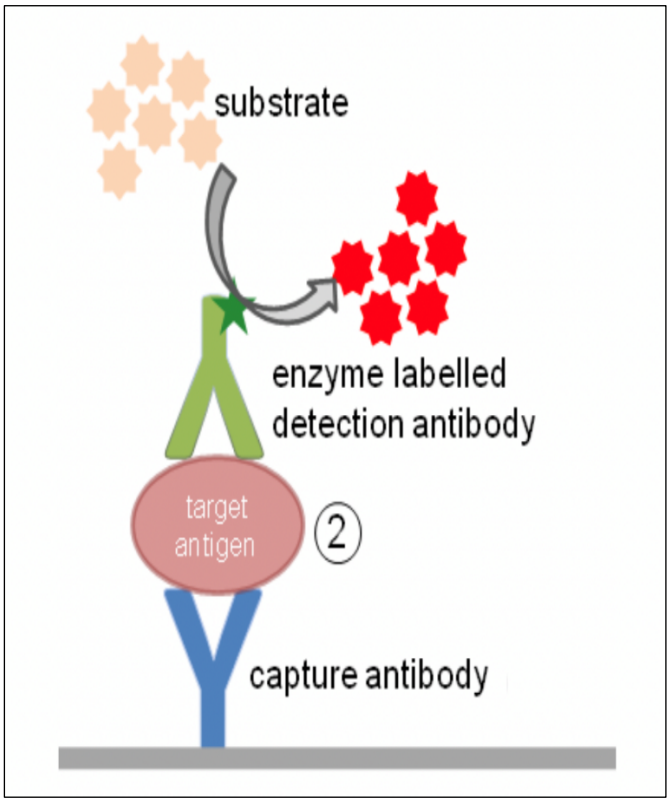

Figure 3.4. A typical enzyme-linked immunosorbent assay (ELISA) sandwich. *Adapted from https://www.immunology.org/public-information/bitesized-immunology/experimental-techniques/enzyme-linked-immunosorbent-assay.*

3.7.2. The procedure of IL-6 ELISA

The reagents contained in the rat IL-6 ELISA kit (Rat IL-6 Immunoassay, # R6000B Quantikine® ELISA, R&D Inc., MN) were prepared as per the manufacturer's protocol.

First, 50µL of assay diluent was added to each well in the assay plate, and then 50µL of standards, 50µL of each sample, and 50µL of assay control were added in their designated wells in the plate. Subsequently, the plate was incubated at room temperature (25°C) for 2 hours.

After incubation, the plate was washed five times with wash buffer and 10µL of rat IL-6 conjugate was added to each well.

The plate was incubated again for 2 hours at room temperature. After incubation, the rat IL-6 conjugate was aspirated, and each well was washed five times with wash buffer. 100µL of substrate solution was added to each well, and the plate was incubated under darkness for 30 minutes.

At the end of the 30 minutes, the stop solution was added to each well, and the plate was read by a Biotek microplate reader (Biotek-Synergy H1 Hybrid Reader, VT) at 450 nm.[256]

3.8. ROS assay

3.8.1. The principle of ROS assay

The ROS assay employs a fluorogenic dye named 2',7'–dichlorofluorescein diacetate (DCFDA).[257] It measures the free radicals such as hydroxy, peroxyl and other ROS within a cell. The assay principle is based on the process of deacetylation of DCFDA. The dye readily diffuses into cells and gets deacetylated into a 'non-fluorescent' compound by cellular esterase enzymes present in cells. The so formed non-fluorescent compound later gets oxidized by ROS present in cells into a 'highly fluorescent' compound called 2',7'–dichlorofluorescein (DCF) which is detected by fluorescence spectroscopy with maximum excitation at 485 nm and emission at 535 nm.[258]

3.8.2. ROS assay procedure

The ROS assay was carried out as per the manufacturer's protocol (# ab113851, DCFDA Cellular ROS Detection Assay Kit, Abcam®, MA). Aortic VSMCs were seeded at 1×10^4 cells/well in 96-well plates, cultured and made quiescent by replacing serum media with SFM 24 hours prior to treatment. On the day of the experiment, cells were first washed with sterile PBS and then stained with 100 µL of DCFDA (concentration of 25 µM/well) for 45 minutes under standard incubation conditions.

After incubation, the cells were re-washed with sterile PBS, and 100 µl of SFM was added to each well, and the treatment was carried out as per the experimental protocol. Post-treatment, the 96-well plates were read using a Biotek

microplate reader [(excitation at 485 nm and Emission at 535 nm), Biotek-Synergy H1 Hybrid Reader, VT].

3.9. Western blotting

3.9.1. The principle of Western blotting

Western blotting is also known as immunoblotting. It is a reliable and sensitive technique routinely employed in research for the detection and characterization of proteins. It is a qualitative and semi-quantitative method. Western blotting involves the electrophoretic separation of protein samples based on their size and molecular weight in a polyacrylamide gel. The proteins are electrically transferred from a gel to a nitrocellulose membrane. The electrical transfer of proteins from gel-to-membrane is called 'blotting.'

Following the transfer, membranes are probed with a specific primary antibody and a secondary antibody conjugated with an enzyme (horseradish peroxidase) for the chemiluminescence detection of protein(s).[259] The various steps in Western blotting is shown schematically in **Figure 3.5**.

3.9.2. Preparation of cell lysate and procedure of Western blotting

Cultured aortic VSMCs were lysed on ice with lysis buffer [sodium chloride (NaCl) 137 mM; Tris- hydrochloric acid (HCl) 20 mM; glycerol 10% v/v; Triton X-100 1% v/v; protease inhibitor cocktail—1 tablet per10 mL (Pierce™ Protease Inhibitor, Thermo Scientific, IL)], and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatants were collected, and the bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit, Thermo Scientific, IL) was carried out for estimating total protein content.

Subsequently, samples were diluted with a modified laemmli sample buffer [2.5% SDS, 20% glycerol, 10% beta-mercaptoethanol, 0.004% bromphenol blue,

0.125 M Tris HCl, and pH 6.8 (Bio-Rad, CA)]. The preheated protein samples (equivalent to 20 µg proteins off each sample) were loaded in each well in 10% polyacrylamide gel and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Nitrocellulose membranes were used to transfer the proteins from the gels. The membranes were blocked with 5% w/v non-fat milk (Blotting-Grade Blocker, Bio-Rad, CA) solution [in 0.1% Tween in Tris-buffer saline (TBST)] for one hour at room temperature (25°C).

The membranes were washed three times for five minutes with 0.1% TBST solution and were incubated with primary antibodies overnight at 4°C. After that, the membranes were probed with secondary antibodies conjugated with horseradish peroxidase for one hour at room temperature (25°C). The enhanced chemiluminescence (ECL) reagents (ECL Femto Max, Thermo Scientific, IL) were utilized for visualizing the membranes under chemiluminescence.

The bands were quantified with the Image J Software (National Institutes of Health, MD). The membranes were stripped with the stripping buffer (Restore™ Western Blot Stripping Buffer, VWR Life Science, GA), washed, and blocked in a similar manner as described above, and were incubated with primary antibodies for the housekeeping protein [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], probed with secondary antibodies, and visualized under chemiluminescence and quantified as stated previously.[260,261]

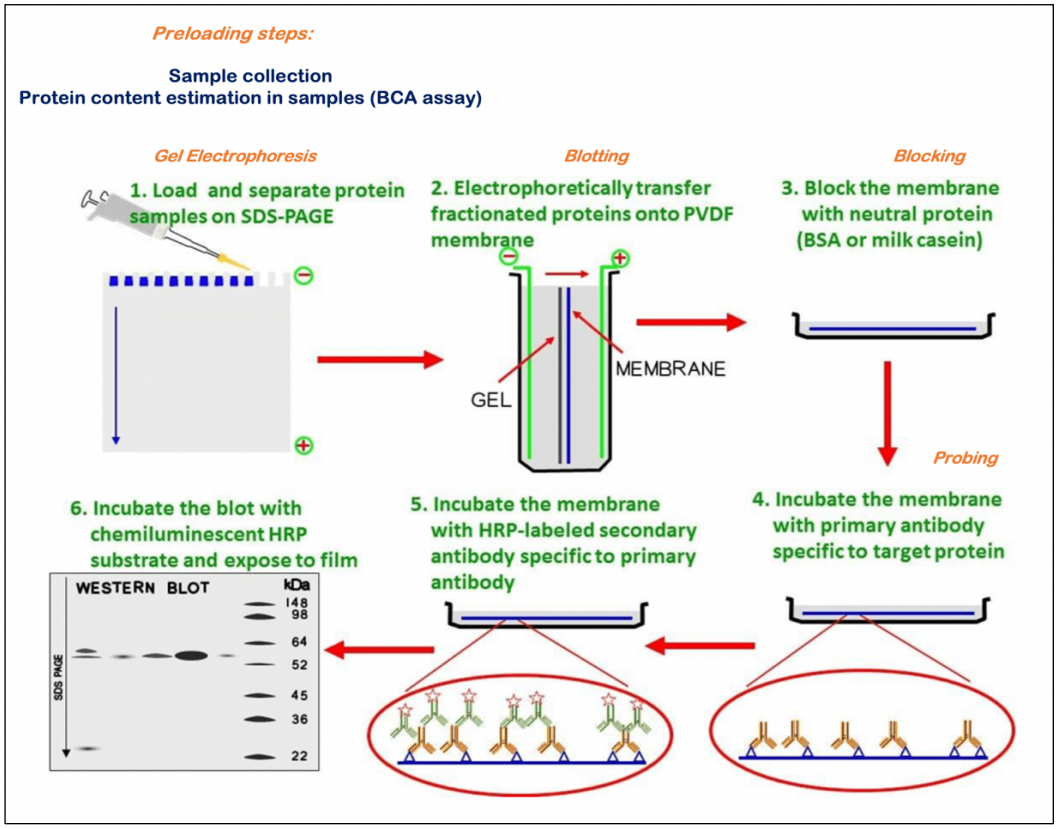

Figure 3.5. A schematic representation of the various steps in Western blotting. *Adapted from* https://microbeonline.com/western-blot-technique-principle-procedures-advantages-and-disadvantages/

The primary and secondary antibodies employed in Western blotting are mentioned in the following table:

Table 3.1: Primary and secondary antibodies used in Western blotting

#	*Antibody*	*Code #*	*Manufacturer*
1	p44/42 MAPK (ERK1/2) (137F5) Rabbit mAb	4695	Cell Signaling Technology®, Inc., MA
2	Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)	9101	Cell Signaling Technology®, Inc., MA
3	ROCK-1 (C8F7) Rabbit mAb	4035	Cell Signaling Technology®, Inc., MA
4	ROCK-2 Antibody	8236	Cell Signaling Technology®, Inc., MA
5	PI3-Kinase Flour® 680 p110 Antibody (D-4) Mouse	sc-8010 AF 680	Santa Cruz Biotechnology Inc., TX
6	Phospho-AKT (Ser473) 587F11 Mouse mAb	4051	Cell Signaling Technology®, Inc., MA
7	GAPDH (D16H11) XP® Rabbit mAb HRP conjugate	8884	Cell Signaling Technology®, Inc., MA
8	Anti-Rabbit IgG HRP-linked Antibody	7074	Cell Signaling Technology®, Inc., MA

9	Anti-Mouse IgG (Fab specific)-Peroxidase Conjugate	A3682	Sigma-Aldrich®, MO

The concentrations of primary and secondary antibodies used in Western blotting are mentioned in the following table:

Table 3.2. Concentrations of primary and secondary antibodies used in Western blotting

#	*Antibody*	*Dilution*	*Function*
1	p44/42 MAPK (ERK1/2)	1:1000 in TBST	Proliferation marker
2	Phospho-p44/42 MAPK (ERK1/2)	1: 1000 in TBST	Proliferation marker
3	ROCK-1	1: 1000 in TBST	Vasocontraction marker
4	ROCK-2	1: 1000 in TBST	Vasocontraction marker
5	PI3-Kinase p110α	1:200 in TBST	Proliferation marker
6	Phospho-AKT	1: 1000 in 5% w/v non-fat dry milk, 1x TBS, and 0.1% Tween-20	Proliferation marker
7	GAPDH	1: 1000 in TBST	Loading control (housekeeping)
8	Anti-Rabbit IgG HRP-linked Antibody	1: 10000 in TBST	Secondary antibody

9	Anti-Mouse IgG (Fab specific)-Peroxidase Conjugate	1: 10000 in TBST	Secondary antibody

The compositions of buffers and polyacrylamide gels: separating and stacking gels used in Western blotting are mentioned in **Table 3.3**, **Table 3.4**, and **Table 3.5**, respectively.

Table 3.3: Composition of buffers used in Western blotting

Buffers	**10X**
Running buffer (for gel electrophoresis)	Tris: 15.20 g Glycine: 72.02 g Sodium dodecyl sulfate (SDS): 5.00 g *Make the volume up to 1,000 mL with distilled water*.
Transfer buffer (for blotting)	Tris: 15.20 g Glycine: 72.02 g *Make the volume up to 1,000 mL with distilled water.*
Tris-buffered saline (TBS)	Tris: 60.57g Sodium chloride (NaCl): 87.66 g *Make the volume up to 1,000 mL with distilled water.*
Phosphate-buffered saline (PBS)	Sodium chloride (NaCl): 82.00 g Potassium chloride (KCl): 2.00 g Disodium hydrogen phosphate (Na_2HPO_4): 29.00 g Potassium dihydrogen phosphate (KH_2PO_4): 2.00 g

	Make the volume up to 1,000 mL with distilled water.

Table 3.4: Composition of 10% resolving (separating) gel used in Western blotting

Ingredients	**2 gels**	**4 gels**
Acrylamide (30%)	3.75 mL	7.50 mL
SDS (10%)	112.50 µL	225.00 µL
Tris-HCl (1.5 M, pH 8.8)	2.85 mL	5.70 mL
Distilled water	4.65 mL	9.3 mL
Ammonium per sulfate [(APS), 10%]	37.50 µL	75 µL
Tetramethylethylenediamine (TEMED)	7.50 µL	15 µL

Table 5: Composition of 5% stacking gel used in Western blotting

Ingredients	**2 gels**	**4 gels**
Acrylamide (30%)	487.50 µL	975.00 µL
SDS (10%)	37.50 µL	75.00 µL
Tris-HCl (1.5 M, pH 8.8)	937.50 µL	1875.00 µL
Distilled water	2.25 mL	4.50 mL
Ammonium per sulfate [(APS), 10%]	19.00 µL	38.00 µL

Tetramethylethylenediamine	4.00 µL	8.00 µL

3.10. Statistical Analysis

The analysis of variance (ANOVA) was used for comparison between Wistar rats and SHRs. The unpaired Student t-test was applied to determine whether differences between the two individual treatment groups were significant. The difference between the treatment groups was considered statistically significant if the probability value (P value) was less than 0.05.[262]

3.11. Specific aims 1 and 2, strategies, and experimental design

Specific aim 1: To characterize the effect of oxytocin on proliferation, inflammation, oxidative stress, and on vasocontraction pathway in primary aortic VSMCs obtained from normotensive Wistar rats.

Specific aim 2: To determine the interaction between oxytocin and Ang II with regards to proliferation, inflammation, oxidative stress, and on vasocontraction pathway in primary aortic VSMCs obtained from normotensive Wistar rats.

Strategies and experimental design for specific aims 1 and 2:

The following four strategies and experimental designs were devised for specific aims 1 and 2:

Strategy 1a:

To investigate the effect of oxytocin, and its interaction with Ang II on the proliferation of aortic VSMCs obtained from normotensive Wistar rats, the following techniques were utilized:

1. Cells were counted directly by employing the Hemocytometer method.
2. Cell viability was assessed using the MTT assay.
3. Cell growth was determined using the ^{3}H-Thymidine incorporation assay.
4. Cell apoptosis and necrosis were detected by Flow Cytometry.

Experimental design for the strategy 1a:

VSMCs were seeded in 6-well plates, 24-well plates, and 96-well plates in the hemocytometer experiments, ^{3}H-Thymidine incorporation assays, and MTT assays, respectively. For the hemocytometer method, MTT-assay, and ^{3}H-

Thymidine incorporation assay, the treatment groups were identical and are stated in the following table:

Table 3.6: Treatment groups in Hemocytometer method, MTT, and ^{3}H-Thymidine incorporation assays

#	**Treatment Groups**
1	The control group (only SFM and no treatment)
2	Oxytocin 100 nM
3	Ang II 100 nM
4	Oxytocin (100 nM) + Ang II (100 nM)
5	L-371,257 [oxytocin receptor antagonist (OXTA)] 1 μM
6	OXTA 1 μM + Oxytocin (100 nM) + Ang II (100 nM)
7	OXTA 1 μM + Oxytocin (100 nM)

Cells were pre-treated with oxytocin (VWR Life Science, GA) ten minutes before treatment with Ang II (VWR Life Science, GA). OXTA (Santa Cruz Biotechnology Inc., TX) was added 30 minutes before the treatment with oxytocin. In both the hemocytometer method and MTT assay, the treatment was carried out every 24 hours through 72 hours. In the ^{3}H-Thymidine incorporation assay, the treatment was carried out at 24 and 48 hours, respectively. The SFM was replenished (every 24 hours) before the start of treatments.

For the flow cytometer assay, 6-well plates were used to seed cells. There were three experimental groups: control (only SFM and no treatment), oxytocin (10 nM), and oxytocin (100 nM). The treatment was carried out only for 24 hours.

Strategy 1b:

To further understand the mechanism of oxytocin, and its interaction with Ang II on the proliferation of aortic VSMCs in Wistar rats, the two critical signaling pathways involved in VSMCs proliferation were investigated: ERK1/2 pathway and PI3K/Akt pathway. Western blotting technique was utilized as a part of strategy 1b.

Experimental design for Strategy 1b:

Cells were seeded in 6-well plates. The experimental design consisted of the following treatment groups mentioned in **Table 3.7**:

Table 3.7: Treatment groups for ERK1/2 and PI3K/Akt pathway experiments

#	**Treatment Groups**
1	The control group (only SFM and no treatment)
2	Oxytocin 100 nM
3	Ang II 100 nM
4	Oxytocin (100 nM) + Ang II (100 nM)
5	L-371,257 [oxytocin receptor antagonist (OXTA)] 1 μM
6	OXTA 1 μM + Oxytocin (100 nM) + Ang II (100 nM)

For investigating the effect of oxytocin, and its interaction with Ang II on the phosphorylation of ERK1/2, PI3K p110α, and Akt, cells were treated with oxytocin for ten minutes, and also with Ang II also for ten minutes. In the combination group, cells were pre-treated with oxytocin for ten minutes before the

treatment with Ang II. OXTA was added 30 minutes before the treatment with oxytocin.

Strategy 2:

To investigate the effect of oxytocin, and its interaction with Ang II on oxidative stress in aortic VSMCs (Wistar rats), ROS levels were measured. A ROS assay was carried out to determine ROS levels.

Experimental design for strategy 2:

Cells were seeded in 96-well plates. The experimental design for the ROS assay comprised of the following treatment groups (**Table 3.8)**:

Table 3.8: Treatment groups for the ROS assay

#	**Treatment Groups**
1	The control group (SFM and no treatment)
2	Oxytocin 100 nM
3	Ang II 100 nM
4	Oxytocin (100 nM) + Ang II (100 nM)
5	L-371,257 [oxytocin receptor antagonist (OXTA)] 1 μM
6	OXTA 1 μM + Oxytocin (100 nM) + Ang II (100 nM)
7	OXTA 1 μM + Oxytocin (100 nM)
8	OXTA 1 μM + Ang II (100 nM)

Cells were treated for ten minutes with oxytocin and Ang II. In the combination group, cells were pre-treated with oxytocin ten minutes before the

treatment with Ang II. OXTA was added 30 minutes before the treatment with oxytocin and Ang II.

Strategy 3:

To investigate the effect of oxytocin, and its interaction with Ang II on inflammation in aortic VSMCs (Wistar rats), the levels of IL-6, a critical inflammatory mediator, were measured. An IL-6 ELISA was utilized to measure IL-6 levels.

Experimental design for strategy 3:

Cells were seeded in 6-well plates. The experimental design for the IL-6 assay included the following treatment groups (**Table 3.9)**:

Table 3.9: Treatment groups for the IL-6 assay

#	**Treatment Groups**
1	The control group (only SFM and no treatment)
2	Oxytocin (10 nM)
3	Oxytocin (100 nM)
4	Oxytocin (1000 nM)
5	Ang II (100 nM)
6	Oxytocin (10 nM) + Ang II (100 nM)
7	Oxytocin (100 nM) + Ang II (100 nM)
8	Oxytocin (1000 nM) + Ang II (100 nM)
9	L-371,257 [oxytocin receptor antagonist (OXTA)] + Oxytocin (100 nM) + Ang II (100 nM)

Cells were treated with different concentrations of oxytocin for ten minutes, and with Ang II for ten minutes. In the combination group, cells were pre-treated with oxytocin ten minutes before the treatment with Ang II. OXTA was added 30 minutes before the treatment with oxytocin and Ang II. Post-treatment, IL-6 secreted in the media (supernatant) was collected, and IL-6 ELISA was carried out as per the manufacturer's protocol.

Strategy 4:

The effect of oxytocin, and its interaction with Ang II on vasocontraction in aortic VSMCs in Wistar rats was assessed indirectly by investigating the Rho-kinase pathway (ROCK-1 and ROCK-2) a critical pathway implicated in vasocontraction of VSMCs. The Western blotting method was employed as part of strategy 4.

Experimental design for strategy 4:

Cells were seeded in 6-well plates. The experimental design to pursue strategy 4 incorporated the following treatment groups (**Table 3.10**):

Table 3.10: Treatment groups for Rho kinase pathway experiments

#	**Treatment Groups**
1	The control group (only SFM and no treatment)
2	Oxytocin 100 nM
3	Ang II 100 nM
4	Oxytocin (100 nM) + Ang II (100 nM)
5	L-371,257 [oxytocin receptor antagonist (OXTA)] 1 μM
6	OXTA 1 μM + Oxytocin (100 nM) + Ang II (100 nM)

To determine the effect of oxytocin, and its interaction with Ang II on the phosphorylation of ROCK-1 and ROCK-2, cells were treated with oxytocin for ten minutes, and with Ang II also for ten minutes. In the combination group, cells were pre-treated with oxytocin for ten minutes before the treatment with Ang II. OXTA was added 30 minutes before the treatment with oxytocin.

3.12. Specific aim 3, strategy, and experimental design

Specific aim 3: To characterize the effect of oxytocin and its interaction with Ang II on proliferation, inflammation, oxidative, and vasocontraction pathway in primary aortic VSMCs obtained from SHRs.

Strategies and experimental design for specific aim 3:

To pursue specific aim 3, the approaches similar to specific aims 1 and 2 were adopted, and identical experimental designs were employed in aortic VSMCs obtained from hypertensive rats (SHRs).

Chapter 4: Results

4.1. Normotensive Wistar rats

(Specific aims 1 and 2)

4.1.1. Effects of oxytocin and Ang II on proliferation of aortic VSMCs isolated from normotensive Wistar rats

4.1.1.1. Cell counting by Hemocytometer method in Wistar VSMCs

Oxytocin (100 nM) significantly reduced the proliferation of aortic VSMCs compared to the control group in normotensive Wistar rats. Oxytocin-induced reduction of VSMCs proliferation rate of VSMCs was time-dependent, with a 50.9% ± 8.3% ($P < 0.05$) reduction in proliferation at 24 hours, 89.2% ± 1.2 % ($P < 0.001$) reduction at 48 hours and 94.7% ± 5.3% ($P < 0.001$) at 72 hours compared to control (**Figures 4.1** and **4.2**).

Contrary to oxytocin, Ang II (100 nM) significantly increased the proliferation of VSMCs by 354% ± 46% ($P < 0.001$) at 24 hours, 217% ± 13% ($P < 0.001$) at 48 hours, and 118% ± 73% ($P < 0.001$) at 72 hours when compared to control (**Figures 4.1** and **4.2**).

Pre-treatment with oxytocin (100 nM) for ten minutes remarkably reduced the Ang II-induced increase in VSMCs proliferation by 62.9% ± 8.8% ($P < 0.01$) at 24 hours, 82.9% ± 21.5% ($P < 0.001$) at 48 hours and 86.9% ± 5.5% ($P < 0.001$)

at 72 hours compared to Ang II alone. These effects of oxytocin were prevented by pre-treatment with the OXTA– L-371,257 (1μM), (**Figures 4.1** and **4.2**).

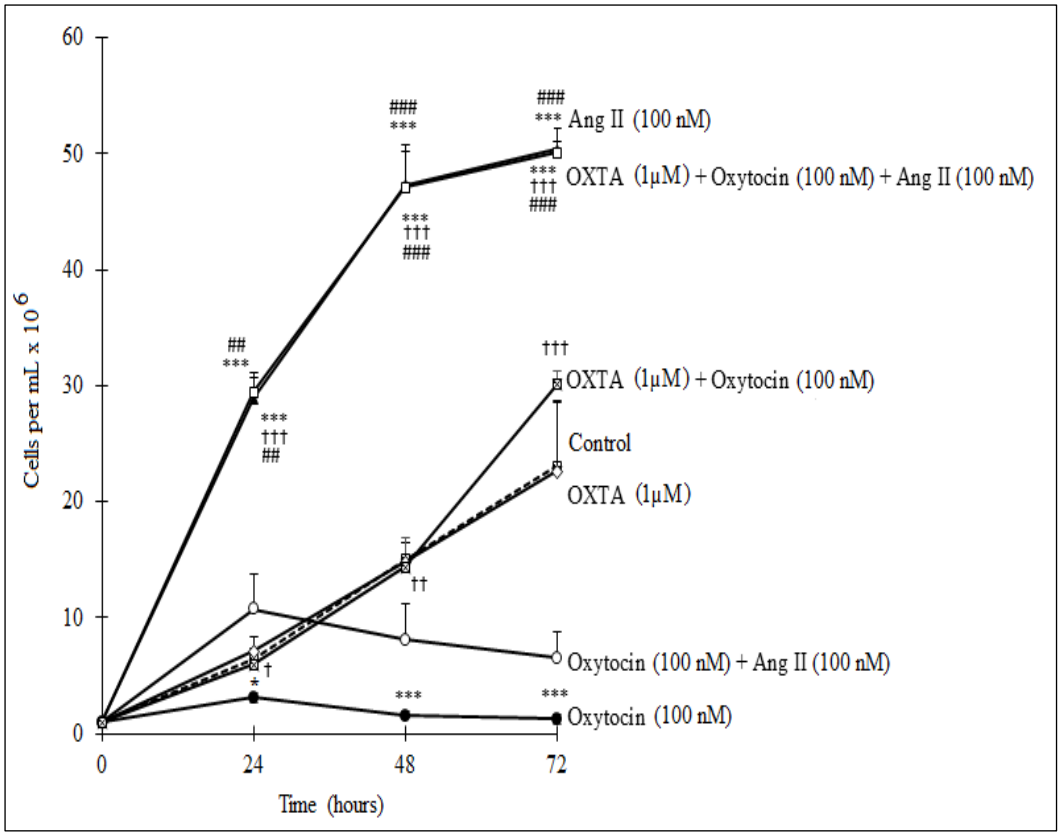

Figure 4.1. The effect of oxytocin and different treatment groups on aortic VSMCs proliferation in Wistar rats as assessed by the Hemocytometer method. *Cells were seeded at 1×10^6 cells/well in 6-well plates. Cell proliferation was measured by counting the number of cells per milliliter (mL). The data are represented as the mean ± SEM from three independent experiments performed in duplicates using aortic VSMCs from three Wistar rats. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

** $P < 0.05$ and *** $P < 0.001$: All groups versus Control*

† $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$: Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

$P < 0.01$ and ### $P < 0.001$: (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

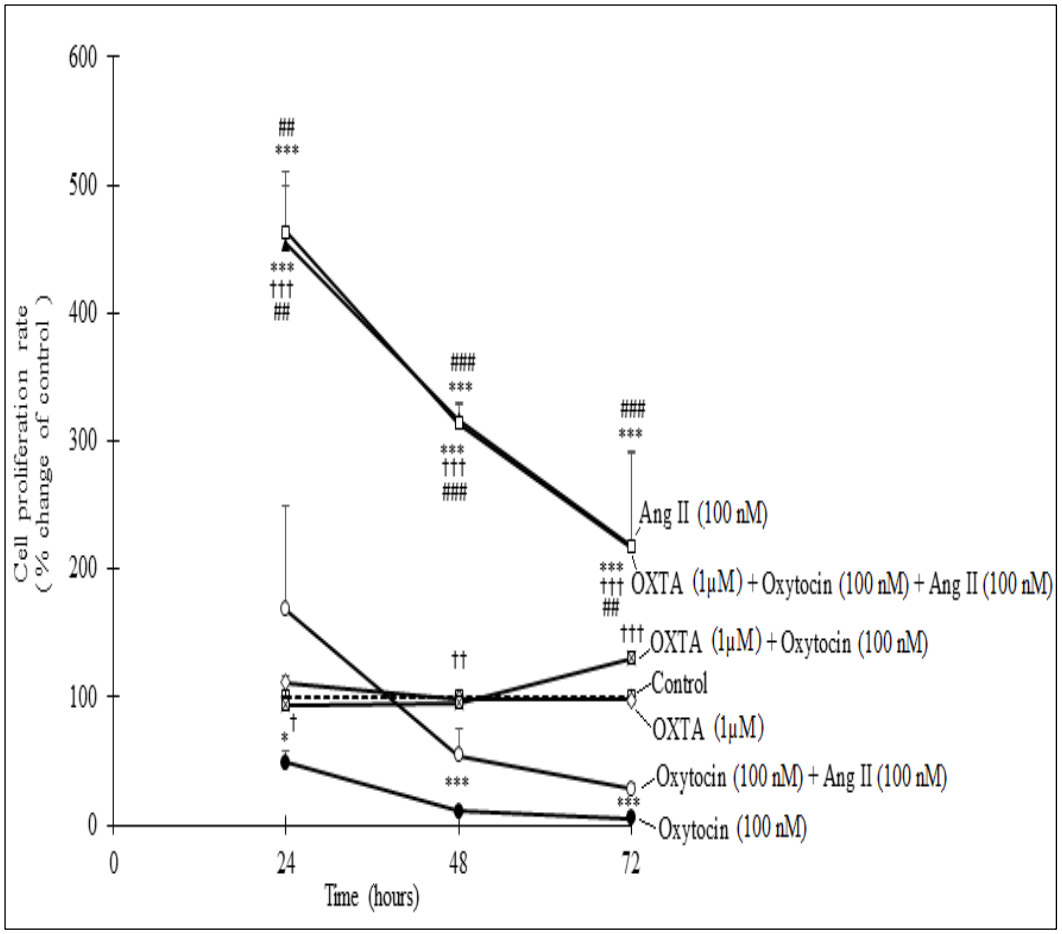

Figure 4.2. The effect of oxytocin and different treatment groups on the proliferation rate of aortic VSMCs in Wistar rats as assessed by the Hemocytometer method. *Cells were seeded at 1×10^6 cells/well in 6-well plates. Cell proliferation rate was assessed as percent (%) change of control. The data are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three Wistar rats. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

** $P < 0.05$ and *** $P < 0.001$: All groups versus Control*

† $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$: Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

$P < 0.01$ and ### $P < 0.001$: (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

4.1.1.2. Assessment of cell viability with the MTT-assay in Wistar VSMCs

The effect of oxytocin on cell viability in aortic VSMCs was evaluated with the MTT assay. Oxytocin (100 nM) induced a significant decrease in cell viability of VSMCs in a time-dependent manner with 4.4% ± 3% ($P < 0.001$), 22.6% ± 7% ($P < 0.001$), and 21.4% ± 6% ($P < 0.001$) reduction at 24, 48 and 72 hours, respectively compared to control (**Figures 4.3 and 4.4**).

Contrary to oxytocin's effect, Ang II (100 nM) treatment showed a significant increase in cell viability of VSMCs with 112% ± 15% ($P < 0.001$), 119% ± 15% ($P < 0.001$), and 79.6% ± 10% ($P < 0.001$) increases in proliferation of VSMCs at 24, 48, and 72 hours, respectively compared to control (**Figures 4.3 and 4.4**).

The pre-treatment with oxytocin (100 nM) also significantly reduced Ang II-induced VSMCs viability with 53.8% ± 4.5% ($P < 0.001$) decrease at 24 hours, 63.8% ± 7.7% ($P < 0.001$) decrease at 48 hours and a 51.6% ± 5.8% ($P < 0.001$) decrease at 72 hours compared to Ang II alone. Both these effects of oxytocin were significantly antagonized by the OXTA (**Figures 4.3 and 4.4**).

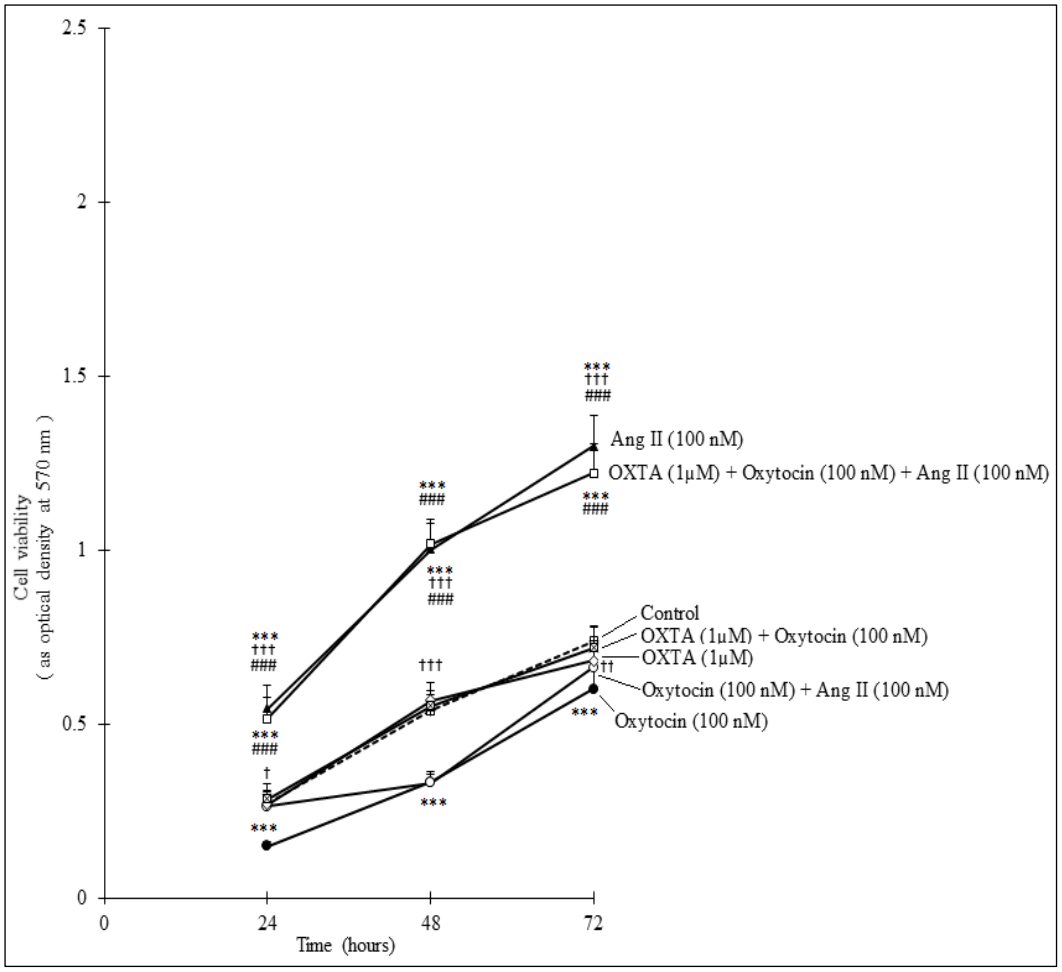

Figure 4.3. The effect of oxytocin and different treatment groups on Wistar aortic VSMCs viability as assessed by the MTT assay. *Cells were seeded at 1×10^4 cells/well in 96-well plates. Cell viability was determined as the function of an optical density (OD) measured at 570 nm. The data are shown as the mean ± SEM of three independent experiments performed using aortic VSMCs isolated from three Wistar rats. Each experiment was performed with 12 replicates. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

*** $P < 0.001$: *All groups versus Control*

† $P < 0.05$, †† $P < 0.01$, *and* ††† $P < 0.001$: *Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II*

$P < 0.001$: *(Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)*

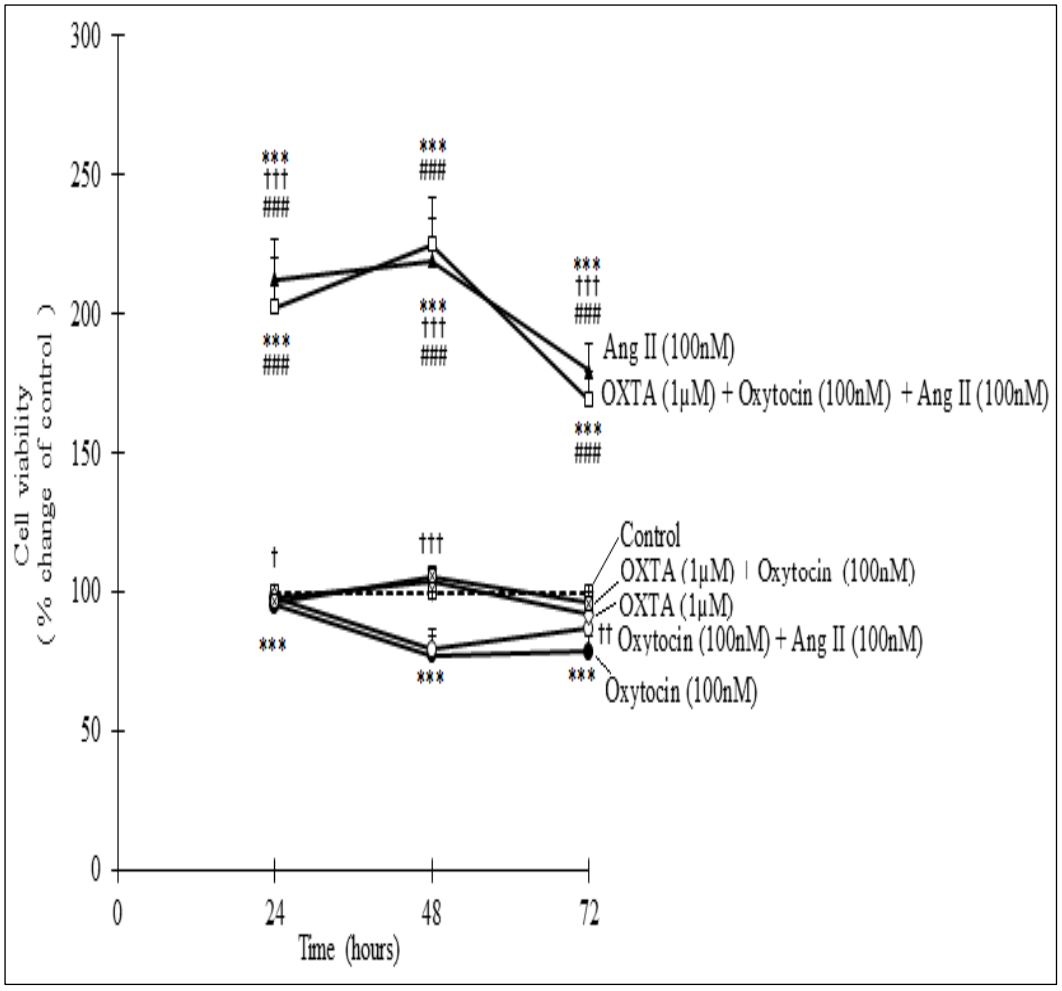

Figure 4.4. The effect of oxytocin and different treatment groups on Wistar aortic VSMCs viability rate as assessed by the MTT assay. *Cells were seeded at 1×10^4 cells/well in 96-well plates. Cell viability was measured as the percent (%) change of control. The data are the shown as the mean ± SEM of three independent experiments performed using aortic VSMCs isolated from three Wistar rats. Each experiment was performed with 12 replicates. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

*** $P < 0.001$: All groups versus Control

† $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$: Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

$P < 0.001$: (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

4.1.1.3. Assessment of cell growth with ^{3}H-Thymidine incorporation assay in Wistar VSMCs

The growth assay allowed an indirect measurement of cell division/growth – as the function of the incorporation of the radioisotope ^{3}H-Thymidine into deoxyribonucleic acid (DNA) of the rapidly dividing cells. The radioactivity recovered from the cells was measured in terms of counts per minute (cpm). A higher count indicated a higher growth rate and vice-versa.

Growth assay results validated that oxytocin (100 nM) significantly reduced the ^{3}H-Thymidine incorporation in aortic VSMCs from 5,857 ± 3,825 cpm (control) to 2,171 ± 1,446 cpm [44% ± 12% decrease ($P < 0.01$), **Figure 4.5**].

On the other hand, treatment with Ang II (100 nM) significantly increased ^{3}H-Thymidine incorporation in VSMCs from 5,857 ± 3,825 cpm (control) to 11,194 ± 7,874 cpm [70% ± 15% increase ($P < 0.01$), **Figure 4.5**].

Ang II-induced growth of VSMCs was significantly decreased by pre-treatment with oxytocin [4,092 cpm ± 2,257 cpm (43% ± 15% decrease), $P < 0.01$] compared to Ang II alone (11,194 cpm ± 7,874 cpm). Both these effects of oxytocin were antagonized by OXTA (Figure **4.5**).

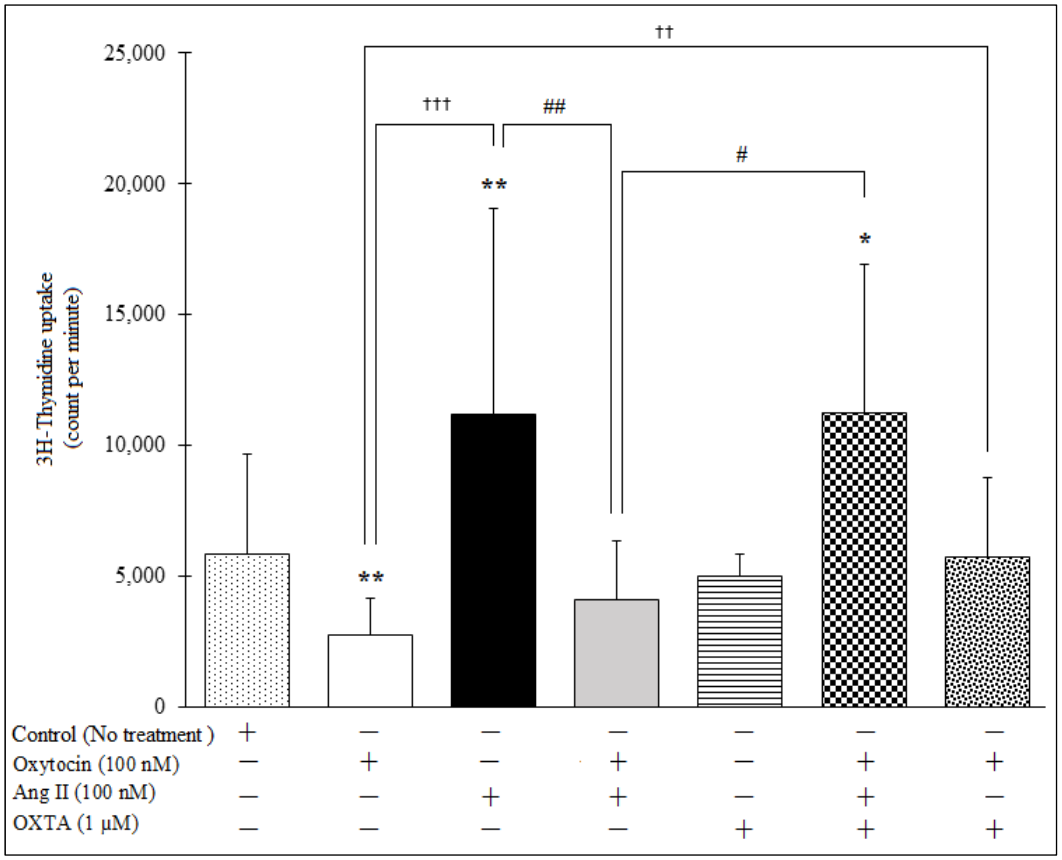

Figure 4.5. The effect of oxytocin and various treatment groups on aortic Wistar VSMCs growth as assessed by the ^{3}H-Thymidine incorporation assay. *VSMCs were seeded at 5×10^{4} cells/well in 24-well plates. Cell growth was measured as the function of the intake of the radioisotope ^{3}H-Thymidine into the deoxyribonucleic acid (DNA) of the rapidly dividing cells and counted by the scintillation counter. The data are represented as the counts per minute (cpm) and are shown as the mean ± SEM of four independent experiments, each performed with triplicates, using aortic VSMCs from four Wistar rats. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

** $P < 0.05$ and ** $P < 0.01$: versus Control*

†† $P < 0.01$ and ††† $P < 0.001$: Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

 # $P < 0.05$ and ## $P < 0.01$: (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II);and (Oxytocin + Ang II) versus and Ang II alone

4.1.1.4. Assessment of cell viability by flow cytometry in Wistar VSMCs

The outcomes of the flow cytometry experiments revealed that treatment with oxytocin decreased cell viability, and induced apoptosis and necrosis in aortic VSMCs isolated from Wistar rats.

The flow experiments utilized two different concentrations of oxytocin: 10 nM and 100 nM. When compared to the control group (95.5% ± 0.7%), the live cell percentage in aortic VSMCs significantly decreased to 91.7 % ± 0.4% ($P < 0.05$) in the 10 nM oxytocin group and further plummeted to 67.5% ± 2.3% ($P < 0.01$) in the 100 nM oxytocin group, respectively (**Figure 4.6)**.

Oxytocin significantly induced apoptosis in aortic VSMCs. The treatment with 100 nM oxytocin significantly increased apoptosis up to 26.1% ± 3% ($P < 0.05$) compared to apoptosis in the control group (2.6% ± 1.1%). However, there were no significant differences in the percentage of apoptotic cells between the 10 nM oxytocin group (4.2% ± 1.6%) and the control group [2.7% ± 1.1% (**Figure 4.6**)].

In addition to inducing apoptosis, the flow results demonstrated that oxytocin considerably induced necrosis in aortic VSMCs. When compared to control (0.7% ± 0.1%), the percentage of cells undergoing necrosis significantly increased to 3.6% ± 1.4% ($P < 0.05$) and 3.9% ± 0.3% ($P < 0.01$) in the10 nM oxytocin group and the 100 nM oxytocin group, respectively. However, there was no statistically significant difference between the percentage of necrotic cells between oxytocin 10 nM an oxytocin 100 nM treatment groups (**Figure 4.6**).

The percentage of aortic VSMCs double positive for both necrosis and apoptosis significantly increased from 0.9% ± 0.1% (in control group) to 2.5% ± 0.1% [in 100 nM oxytocin group ($P < 0.01$)]. Conversely, there were no significant differences between the 10 nM oxytocin group (0.5% ± 0.1%) and the control group [0.9% ± 0.1% (**Figure 4.6**)].

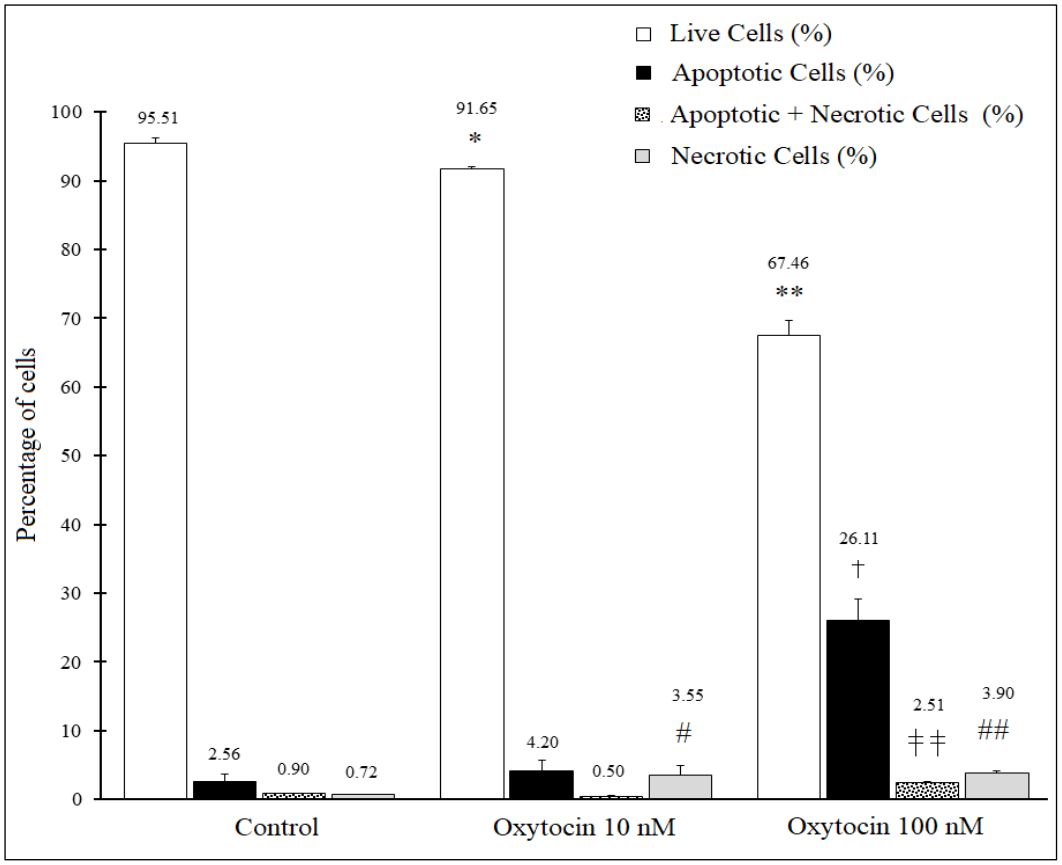

Figure 4.6. The effect of oxytocin on apoptosis and necrosis in aortic Wistar VSMCs as assessed by flow cytometry. *VSMCs were seeded at 1×10^6 cells/well in 6-well plates. The data are shown as the percentage of cells positive for apoptosis, necrosis and apoptosis + necrosis across different treatment groups. The data are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three Wistar rats. The treatment was carried out for 24 hours with the two different concentrations of oxytocin: 10 nM and 100 nM. Cells in the control group were not treated with oxytocin (only SFM). Cells were stained with fluorescent dyes: Annexin V (for apoptosis) and 7-amino actinomycin D [(7-AAD) for necrosis] and assessed with the flow cytometer.*

** $P < 0.05$: Live cells in Oxytocin 10 nM group versus Live cells in Control group*

*** $P < 0.01$: Live cells in Oxytocin 100 nM group versus Live cells in Control group*

† $P < 0.05$: Apoptotic cells in Oxytocin 100 nM group versus Apoptotic cells in Control group

 # P < 0.05 : Necrotic cells in Oxytocin 10 nM group versus Necrotic cells in Control group

P < 0.01 : Necrotic cells in Oxytocin 100 nM group versus Necrotic cells in Control group

‡‡ P < 0.01 : (Apoptotic + Necrotic cells) in Oxytocin 100 nM group versus (Apoptotic + Necrotic cells) in Control group

4.1.2. Effect of oxytocin and its pre-treatment with Ang II on the phosphorylation of ERK1/2 in aortic Wistar VSMCs

In aortic Wistar VSMCs, oxytocin (100 nM) significantly decreased the active phosphorylated form of ERK1/2 (pERK1/2) after ten minutes of treatment compared to the control group (basal levels). While the treatment with Ang II (100 nM) significantly increased pERK1/2 after ten minutes of treatment compared to control (**Figure 4.7**).

The pre-treatment with oxytocin (100 nM) significantly reduced Ang II-induced activation of pERK1/2, and pre-treatment with OXTA (1 μM) for 30 minutes significantly abrogated these effects of oxytocin (**Figure 4.7**).

The densitometric analysis of the western blot images showed that oxytocin significantly decreased pERK1/2 by 57.9% ± 14.2% ($P < 0.001$) compared to control, Ang II significantly increased pERK1/2 by 223% ± 11% ($P < 0.001$) compared to control, and the oxytocin pre-treatment significantly decreased pERK1/2 by 76.6% ± 1.4% ($P < 0.01$) compared to Ang II alone (**Figure 4.7**).

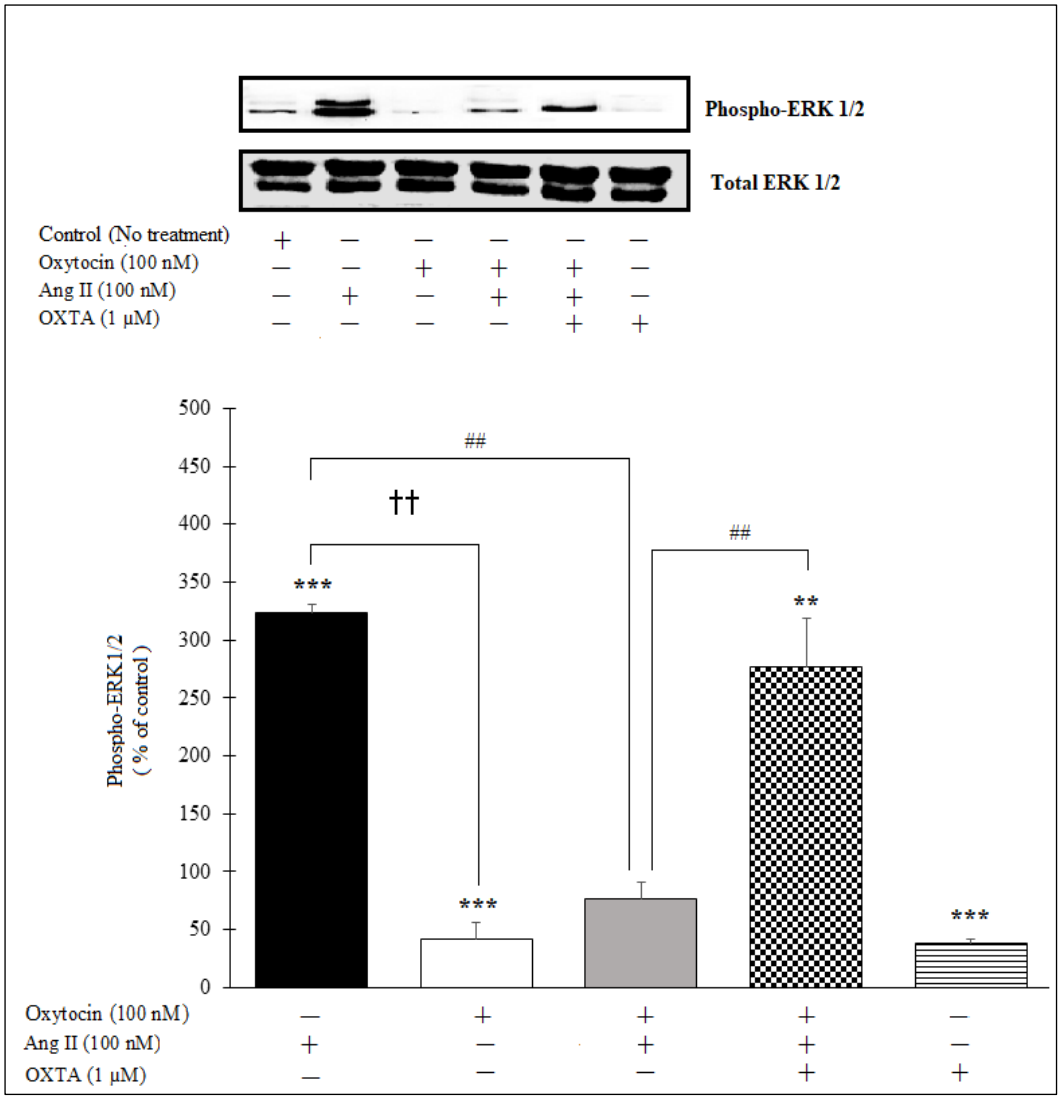

Figure 4.7. The effect of oxytocin and different treatment groups on the phosphorylation of ERK1/2 in aortic Wistar VSMCs. *Western blotting was utilized to measure phospho-ERK1/2 levels, and the data were normalized with total ERK1/2. The effect of oxytocin and its combination with different treatment groups on the phosphorylation of ERK1/2 is shown as the percent (%) of control, with control as 100%. The data are shown as the mean ± SEM of five independent experiments, performed using aortic VSMCs isolated from five Wistar rats. ** indicates $P < 0.01$ versus control; *** $P < 0.001$ versus control; †† $P < 0.01$ Oxytocin versus Ang II; ## $P < 0.01$ (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

4.1.3. Effect of oxytocin and its pre-treatment with Ang II on the phosphorylation of PI3K p110α and Akt in aortic Wistar VSMCs

In primary aortic VSMCs obtained from Wistar rats, the densitometric analysis of the western blot images showed that the treatment with oxytocin (100 nM) significantly decreased the active phosphorylated form of PI3K p110alpha (phospho-PI3K p110α) by 64.8% ± 2.8% ($P < 0.01$) and the active phosphorylated form of Akt (phospho-Akt) by 46.2% ± 9.5% ($P < 0.05$) compared to controls, respectively (**Figures 4.8** and **4.9**).

One the other hand, treatment with Ang II (100 nM) significantly increased the levels of phospho-PI3K p110α by 184% ± 17% ($P < 0.05$) and phospho-Akt by 44.4% ± 3.5% ($P < 0.001$) compared to control respectively (**Figures 4.8** and **4.9**).

Pre-treatment with oxytocin (100 nM) significantly reduced Ang II-mediated increased levels of phospho-PI3K p110α by 83.8% ± 8.6% ($P < 0.01$) and phospho-Akt by 62.1% ± 10.9% ($P < 0.001$) compared to Ang II alone, respectively. These effects of oxytocin were significantly abated by pre-treatment with OXTA (1 μM) for 30 minutes (**Figures 4.8** and **4.9**).

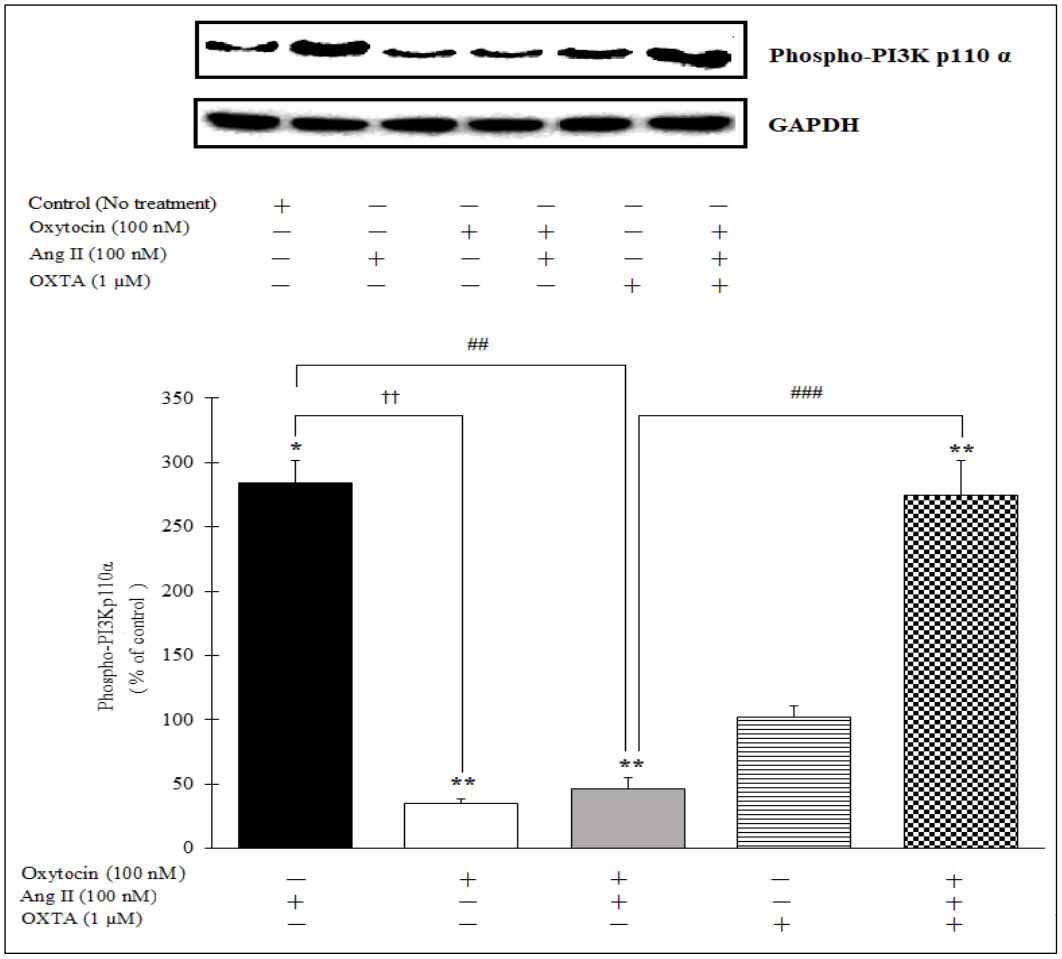

Figure 4.8. The effect of oxytocin and different treatment groups on the phosphorylation of PI3K p110α in aortic Wistar VSMCs. *Western blotting was utilized to measure the phosphorylation of PI3K p110α, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the phosphorylation of PI3K p110α is shown as the percent (%) of control, with control as 100 %. The data are shown as the mean ± SEM of three independent experiments, performed using aortic VSMCs isolated from three Wistar rats. * indicates $P < 0.05$ versus control; ** $P < 0.01$ versus control; †† $P < 0.01$ Oxytocin versus Ang II; ## $P < 0.01$ (Oxytocin + Ang II) versus Ang II alone; ### $P < 0.001$ (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

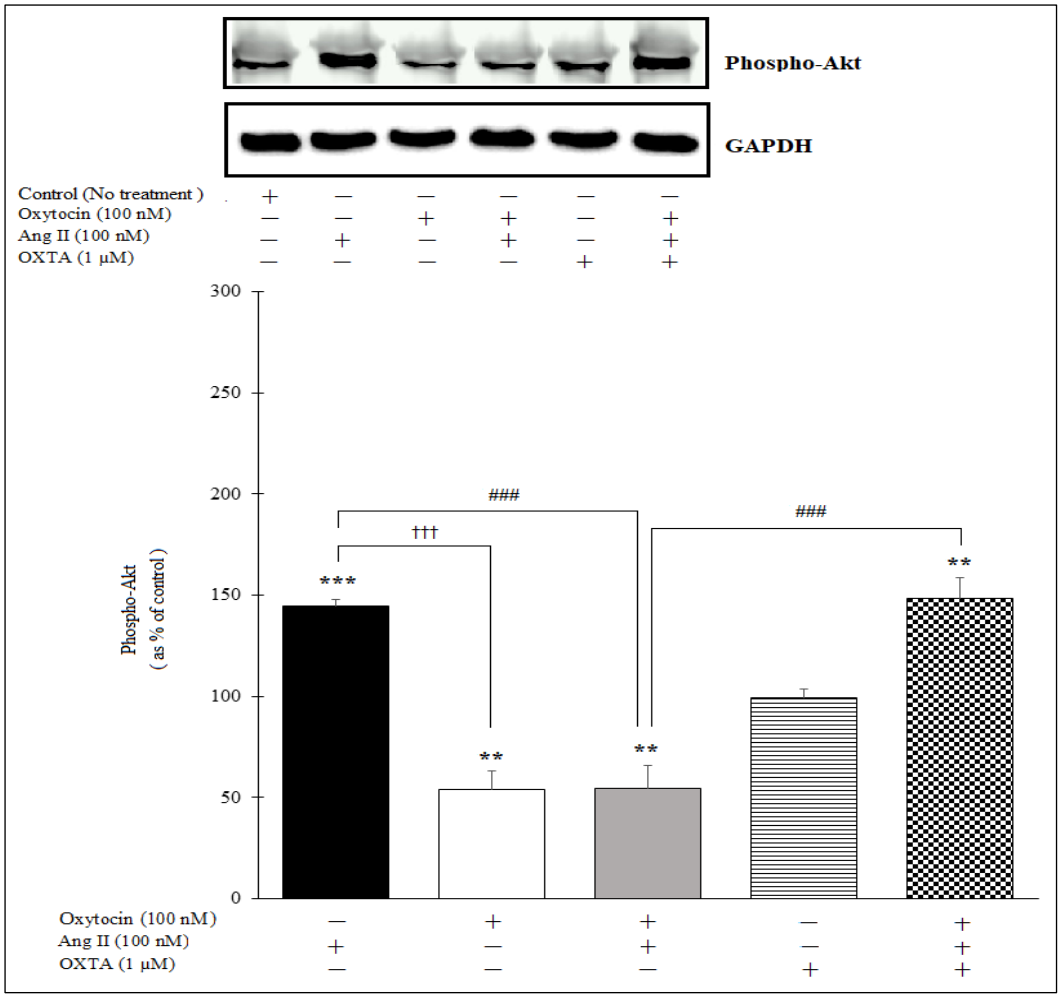

Figure 4.9. The effect of oxytocin and different treatment groups on the phosphorylation of Akt in aortic Wistar VSMCs. *Western blotting was utilized to measure the phosphorylation of Akt, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the phosphorylation of Akt is shown as the percent (%) of control, with control as 100%. The data are shown as the mean ± SEM of four independent experiments, performed using aortic VSMCs isolated from four Wistar rats. ** indicates P < 0.01 versus control; *** P < 0.001 versus control; ††† P < 0.001 Oxytocin versus Ang II; ### P < 0.001 (Oxytocin + Ang II) versus Ang II alone, and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

4.1.4. Effect of oxytocin and its pre-treatment with Ang II on the activation of ROCK -1 and ROCK-2 in aortic Wistar VSMCs

Densitometric analysis of the western blot images showed that in Wistar rats, oxytocin (100 nM) significantly reduced the activation of ROCK-1 by 60.4% ± 7.6% ($P < 0.001$) and ROCK-2 by 38.7% ± 13.1% ($P < 0.01$) compared to controls, respectively (**Figures 4.10** and **4.11**).

Ang II (100 nM) significantly enhanced the activation of ROCK-1 by 147% ± 50% ($P < 0.05$) and ROCK-2 by 83.2% ± 14.6% ($P < 0.001$) compared to controls, respectively (**Figures 4.10** and **4.11**).

Pre-treatment with oxytocin (100 nM) significantly reduced Ang II-induced activation of ROCK-1 by 85.2% ± 10.6% ($P < 0.01$) and ROCK-2 by 65.9% ± 13.2% ($P < 0.001$) compared to Ang II alone, respectively. These effects of oxytocin were significantly reduced by pre-treatment with OXTA (1μM) (**Figures 4.10** and **4.11**).

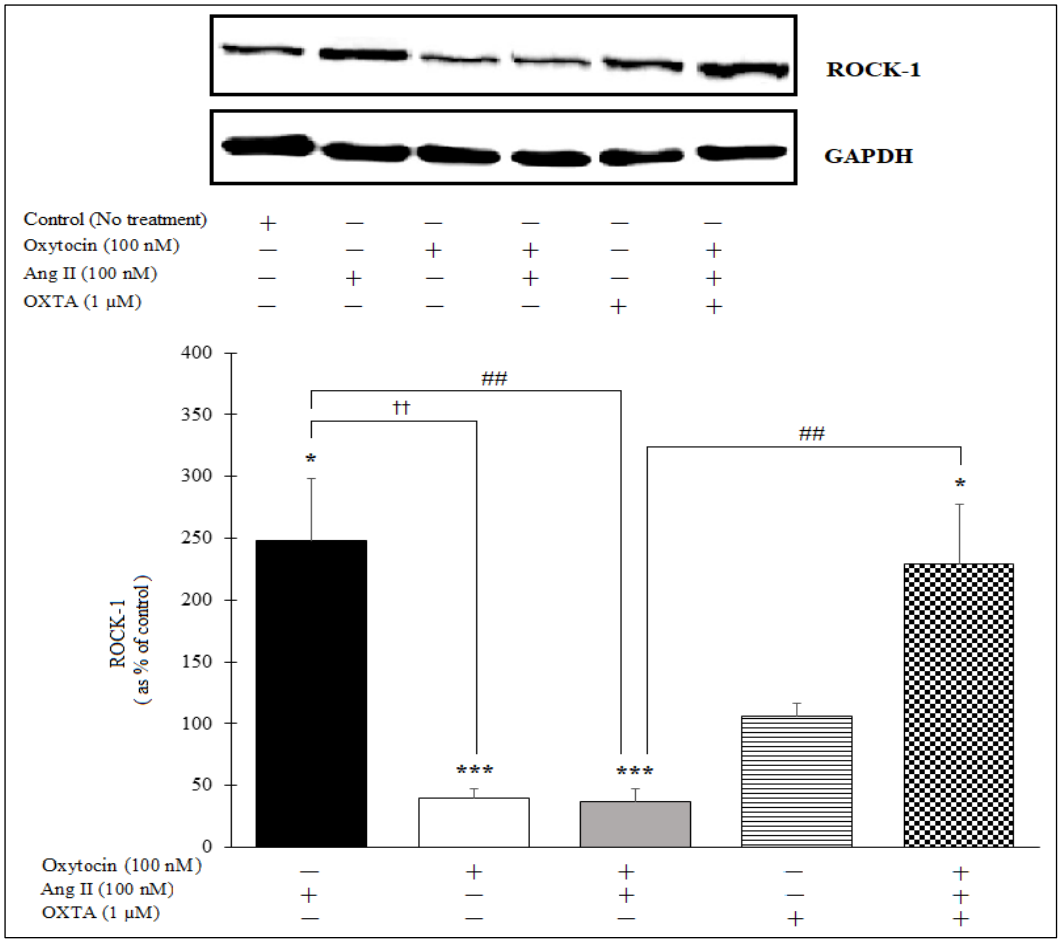

Figure 4.10. The effect of oxytocin and different treatment groups on the activation of ROCK- 1 in aortic Wistar VSMCs. *Western blotting was utilized to measure the ROCK-1 levels, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the activation of ROCK- 1 is shown as the percent (%) of control, with control as 100%. The data are shown as the mean ± SEM of four independent experiments, performed using aortic VSMCs isolated from four Wistar rats. * indicates $P < 0.05$ versus control; *** $P < 0.001$ versus control; †† $P < 0.01$ Oxytocin versus Ang II; ## $P < 0.01$ (Oxytocin + Ang II) versus Ang II alone, and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist; ROCK, Rho-associated coiled-coil containing protein kinase.*

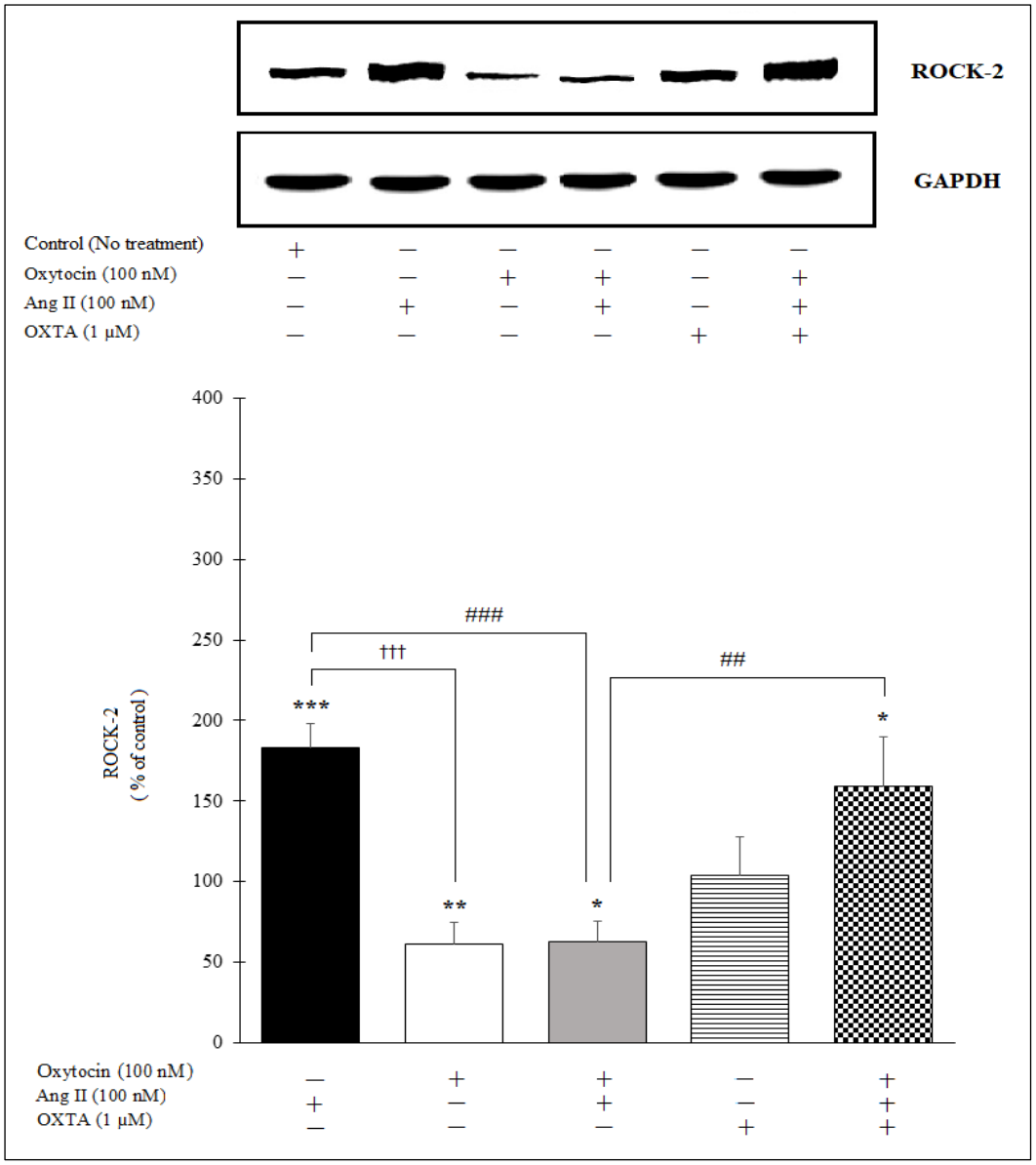

Figure 4.11. The effect of oxytocin and different treatment groups on the activation of ROCK-2 in aortic Wistar VSMCs*. Western blotting was utilized to measure the ROCK-2 levels, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the activation of ROCK-2 is shown as the percent (%) of control, with control as 100%. The data are shown as the mean ± SEM of five independent experiments, performed using aortic VSMCs isolated from five Wistar rats. * indicates P < 0.05 versus control; ** P < 0.01 versus control; *** P < 0.001 versus control; ††† P < 0.001 Oxytocin versus Ang II; ### P < 0.001*

(Oxytocin + Ang II) versus Ang II alone; ## P < 0.01 (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist; ROCK, Rho-associated coiled-coil containing protein kinase.

4.1.5. Effect of oxytocin and its pre-treatment with Ang II on IL-6 secretion in aortic Wistar VSMCs

The IL-6 ELISA assay was carried out as per the manufacturer's protocol. The concentration of IL-6 secreted by aortic VSMCs in the culture media was measured in 'picogram per milliliter' (pg/mL). In Wistar rats, the outcomes of the IL-6 assay showed that oxytocin treatment resulted in a significant increase in IL-6 secretion compared to the control group. Three different concentrations of oxytocin were used: 10 nM, 100 nM, 1,000 nM. The treatment with oxytocin at 100 nM and 1,000 nM resulted in significant increases in IL-6 with 1,033 ± 24 pg/mL ($P < 0.01$) at 100 nM and 959 ± 52 pg/mL ($P < 0.05$) at 1,000 nM, respectively compared to control group [614 ± 104 pg/mL (**Figure 4.12**)].

However, there was no significant difference between oxytocin 10 nM (695 ± 101 pg/mL) and control group (614 ± 104 pg/mL). There was no significant difference between oxytocin 100 nM (1,033 ± 24 pg/mL) and oxytocin 1,000 nM [959 ± 52 pg/mL (**Figure 4.12**)].

Ang II (100 nM) treatment showed a significant increase in IL-6 [1,481 ± 91 pg/mL ($P < 0.01$)] compared to control group (614 ± 104 pg/mL). Ang II (100 nM) treatment resulted in more IL-6 secretion (1,481 ± 91 pg/mL) compared to treatment with oxytocin [100 nM (1,033 ± 24 pg/mL), $P < 0.01$] when compared to control [614 ± 104 pg/mL (**Figure 4.12**)].

Interestingly, in contrast to its singular effect, oxytocin's pre-treatment (100 nM) with Ang II (100 nM) significantly decreased IL-6 secretion (735 ± 77 pg/mL)

compared to Ang II 100 nM alone [1,481 ± 91 pg/mL ($P < 0.01$)] and oxytocin 100 nM alone [1,033 ± 24 pg/mL ($P < 0.01$)], respectively (**Figure 4.12**).

The effect of oxytocin pre-treatment (100 nM) on Ang II (100 nM) was significantly abolished by pre-treatment with 1μM OXTA. IL-6 secretion was significantly higher [1,487 ± 64 pg/mL ($P < 0.001$] in the OXTA-pre-treatment group [OXTA (1μM) + Oxytocin (100 nM) + Ang II (100 nM)] than Oxytocin 100 nM + Ang II 100 nM group [735 ± 77 pg/mL (**Figure 4.12**)].

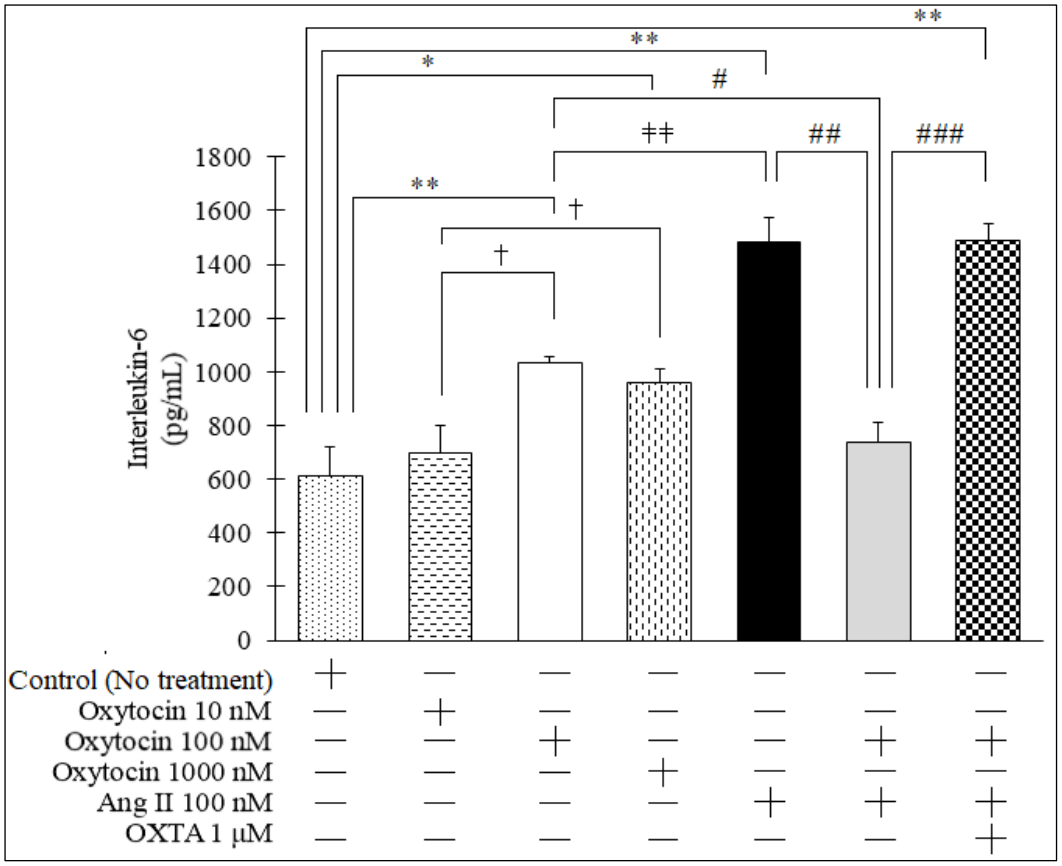

Figure 4.12. The effect of oxytocin and different treatment groups on IL-6 secretion in aortic Wistar VSMCs. *The concentration of IL-6 secreted by aortic VSMCs in the culture media was measured in picogram per milliliter (pg/mL). The data are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three Wistar rats. In each experiment each treatment group had duplicates. * indicates $P < 0.05$ versus Control group; ** $P < 0.01$ versus Control group; † $P < 0.05$ Oxytocin 10 nM versus Oxytocin 100 nM and Oxytocin 1000 nM respectively. $^{\#}P < 0.05$ (Oxytocin 100 nM + Ang II 100 nM) versus Oxytocin 100 nM alone; $^{\#\#}P < 0.01$ (Oxytocin 100 nM + Ang II 100 nM) versus Ang II 100 nM alone; $^{\#\#\#}P < 0.001$ (Oxytocin 100 nM + Ang II 100 nM) versus (OXTA 1µM + Oxytocin 100 nM + Ang II 100 nM); ++ $P < 0.01$ Oxytocin 100 nM versus Ang II 100 nM. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

4.1.6. Effect of oxytocin and its pre-treatment with Ang II on ROS in aortic Wistar VSMCs

ROS was assessed as the function of fluorescent units. The outcomes of the ROS assay in aortic Wistar VSMCs demonstrated that the treatment with oxytocin (100 nM) significantly increased ROS production [8,584 ± 114 ($P < 0.001$)] compared to the control group (7,542 ± 128). Ang II (100 nM) significantly increased ROS production [8,336 ± 204 ($P < 0.001$)] compared to the control group (7,542 ± 128). There was no significant difference in the ROS production between oxytocin [100 nM (8,584 ± 114)] and Ang II [100 nM (8,336 ± 204)] treatment groups (**Figure 4.13**).

The pre-treatment of oxytocin (100 nM) with Ang II (100 nM) significantly increased ROS production [9,346 ± 143 ($P < 0.001$)] compared to the control group [7,542 ± 128 (**Figure 4.13)**].

Interestingly, the pre-treatment of oxytocin (100 nM) followed by Ang II (100 nM) resulted in significantly higher ($P < 0.001$) ROS production (9,346 ± 143) than oxytocin (100 nM) alone (8,584 ± 114) and Ang II (100 nM) alone (8,336 ± 204), respectively, indicating an additive effect (**Figure 4.13**).

OXTA (1 μM) on its own significantly increased ROS production [8,530 ± 267 ($P < 0.001$)] compared to the control group (7,542 ± 128). In addition, pre-treatment of OXTA (1 μM) with oxytocin (100 nM) resulted in significantly increased ROS production [9,594 ± 177 ($P < 0.001$)] when compared with oxytocin (100 nM) alone (8,584 ± 114). This suggests an additive effect of OXTA on oxytocin-stimulated ROS production (**Figure 4.13**).

The combination of OXTA (1 μM), oxytocin (100 nM), and Ang II (100 nM) significantly increased ROS production [10,511 ± 179 ($P < 0.001$)] compared to control (7,542 ± 128) in aortic Wistar VSMCs (**Figure 4.13**).

ROS production in the combination group of OXTA (1 μM), oxytocin (100 nM), and Ang II (100 nM) was significantly higher [10,511 ± 179 ($P < 0.001$)] than Oxytocin (100 nM) alone (8,584 ± 114), Ang II (100 nM) alone (8,336 ± 204), and OXTA (1 μM) alone (8,530 ± 267), respectively, thus indicating an additive effect (**Figure 4.13**).

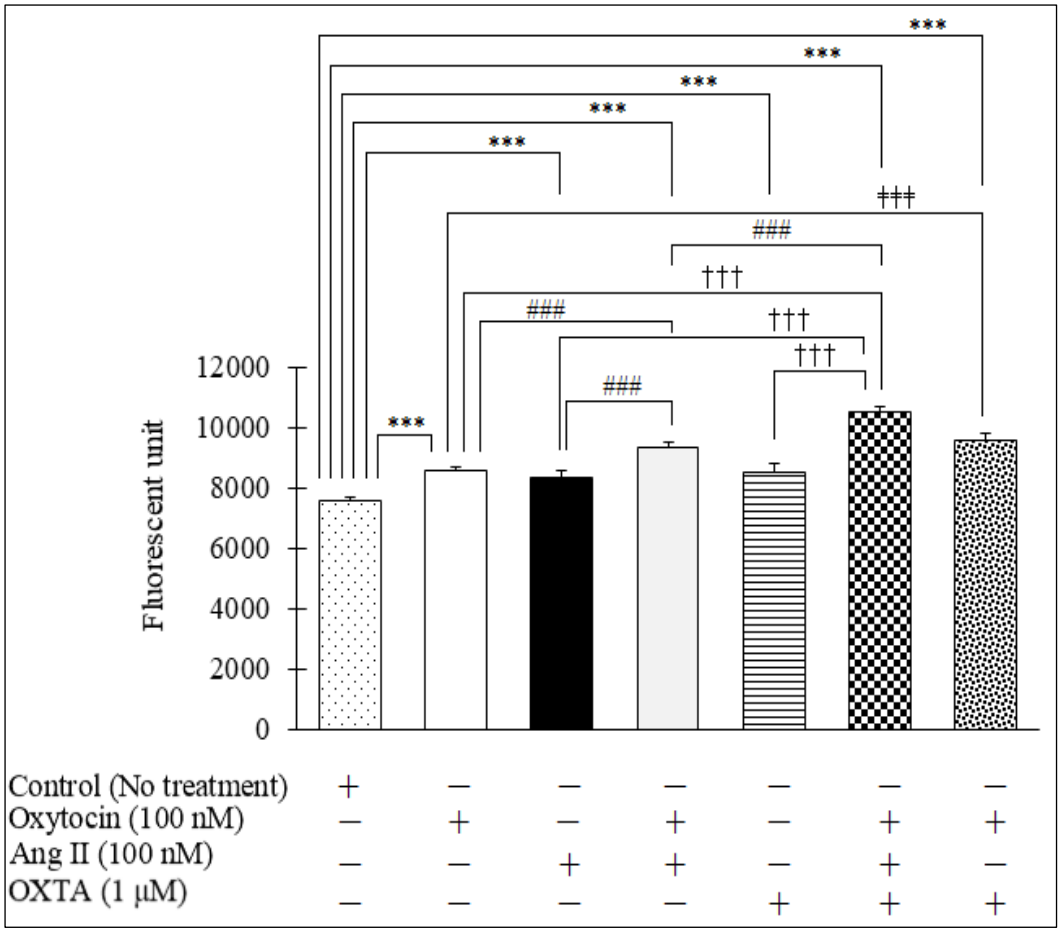

Figure 4.13. The effect of oxytocin and different treatment groups on ROS production in aortic Wistar VSMCs. *VSMCs were seeded at 1×10^4 cells/well in 96-well plates. The ROS levels were measured as the function of the fluorescent unit. The data are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three Wistar rats. In each experiment each treatment group had 12 replicates. *** indicates P < 0.001 versus Control group; ### P < 0.01 (Oxytocin + Ang II) versus Oxytocin alone, Ang II alone, and (OXTA + Oxytocin + Ang II); ††† P < 0.001 (OXTA + Oxytocin + Ang II) versus Oxytocin alone, Ang II alone, and OXTA alone; ‡‡‡ P < 0.001 Oxytocin versus (OXTA + Oxytocin). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

4.2. Spontaneously hypertensive rats [Specific Aim 3]

4.2.1. Effect of oxytocin and its pre-treatment with Ang II on aortic SHR VSMCs proliferation

4.2.1.1. Cell counting by Hemocytometer method in SHR VSMCs

Similar to its effects on VSMCs proliferation from the normotensive rats, oxytocin (100 nM) significantly reduced aortic VSMCs proliferation in SHRs compared to the control group (**Figures 4.14** and **4.15**).

The hemocytometer cell counting revealed that oxytocin-induced reduction in SHR VSMCs proliferation rate was time-dependent, with 44.8% ± 3% reduction ($P < 0.001$) in proliferation at 24 hours, 84.4% ± 0.9% reduction ($P < 0.001$) reduction at 48 hours and 88.2% ± 4.0% reduction ($P < 0.001$) reduction at 72 hours compared to control (**Figures 4.14** and **4.15**).

On the other hand, the treatment with Ang II (100 nM) significantly increased SHR VSMCs proliferation, with 190% ± 18% ($P < 0.001$) increase in proliferation rate at 24 hours, 112% ± 15% ($P < 0.001$) increase at 48 hours, and 207 % ± 56% ($P < 0.001$) increase at 72 hours compared to control (**Figures 4.14** and **4.15**).

It is noteworthy that even in the SHRs, the oxytocin (100 nM) pre-treatment significantly reduced Ang II-induced VSMCs proliferation in a time-dependent manner, with 66% ± 24% ($P < 0.001$) decrease at 24 hours, 93% ± 3% ($P < 0.001$) decrease at 48 hours and 97% ± 4 % ($P < 0.001$) decrease at 72 hours compared to Ang II alone (**Figures 4.14** and **4.15**). Pre-treatment with OXTA (1 μM) significantly diminished these effects of oxytocin (**Figures 4.14** and **4.15**).

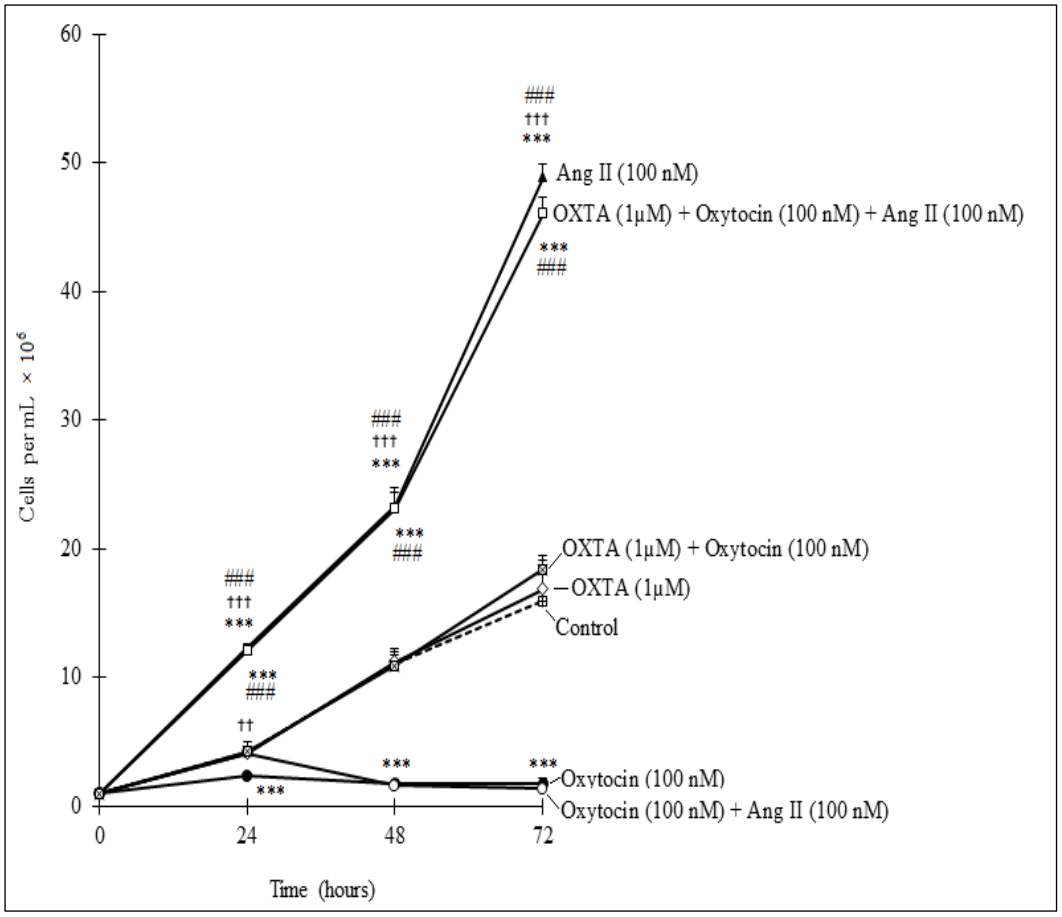

Figure 4.14. The effect of oxytocin and different treatment groups on aortic VSMCs proliferation in SHRs as assessed by the Hemocytometer method. *SHR VSMCs were seeded at 1×10^6 cells/well in 6-well plates. Cell proliferation was measured by counting the number of cells per milliliter (mL). The data are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three SHRs. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

**** $P < 0.001$: All groups versus Control*

†† $P < 0.01$ and ††† $P < 0.001$: Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

$P < 0.001$: (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

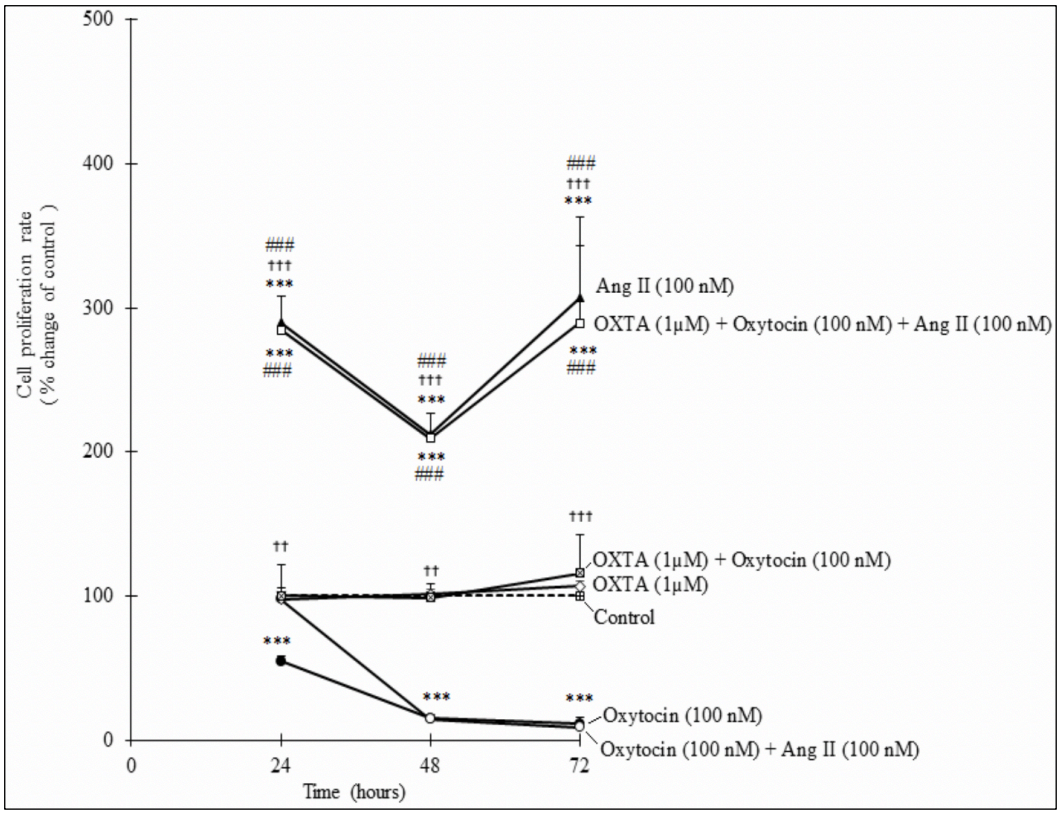

Figure 4.15. The effect of oxytocin and different treatment groups on aortic VSMCs proliferation rate in SHRs assessed by the Hemocytometer method. *SHR VSMCs were seeded at 1×10^6 cells/well in 6-well plates. Cell proliferation rate was assessed as the percent (%) change of control. The data are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three SHRs. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

**** P < 0.001 : All groups versus Control*

†† P < 0.01 and ††† P < 0.001 : Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

P < 0.001 : (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

4.2.1.2. Assessment of cell viability with MTT-assay in SHR VSMCs

The findings from the MTT assay in aortic SHRs VSMCs were similar to those in Wistar rats. Oxytocin (100 nM) significantly reduced cell viability of aortic SHR VSMCs (**Figures 4.16** and **4.17**).

The MTT assay demonstrated that oxytocin caused a reduction in the cell viability of SHR VSMCs by 37.5% ± 3.9% ($P < 0.001$) and 32 % ± 3.8% ($P < 0.001$) at 48 hours and 72 hours, respectively compared to control. However, at 24 hours oxytocin did not show any significant reduction in VSMCs viability rate (**Figures 4.16** and **4.17**).

The treatment with Ang II (100 nM) showed an increase in the VSMCs viability with 145% ± 20% ($P < 0.001$), 121% ± 14% ($P < 0.001$), and 166% ± 13% ($P < 0.001$) increase at 24, 48 and 72 hours, respectively compared to control (**Figures 4.16** and **4.17**).

The pre-treatment with oxytocin (100 nM) prominently reduced Ang II-induced VSMC viability by 51.8% ± 8.3% ($P < 0.001$) at 24 hours, 63.7% ± 5.7% ($P < 0.001$) at 48 hours, and 71.9% ± 3.9% ($P < 0.001$) at 72 hours compared to Ang II alone, and the pre-treatment with OXTA (1 μM) significantly blocked the effects of oxytocin (**Figures 4.16** and **4.17**).

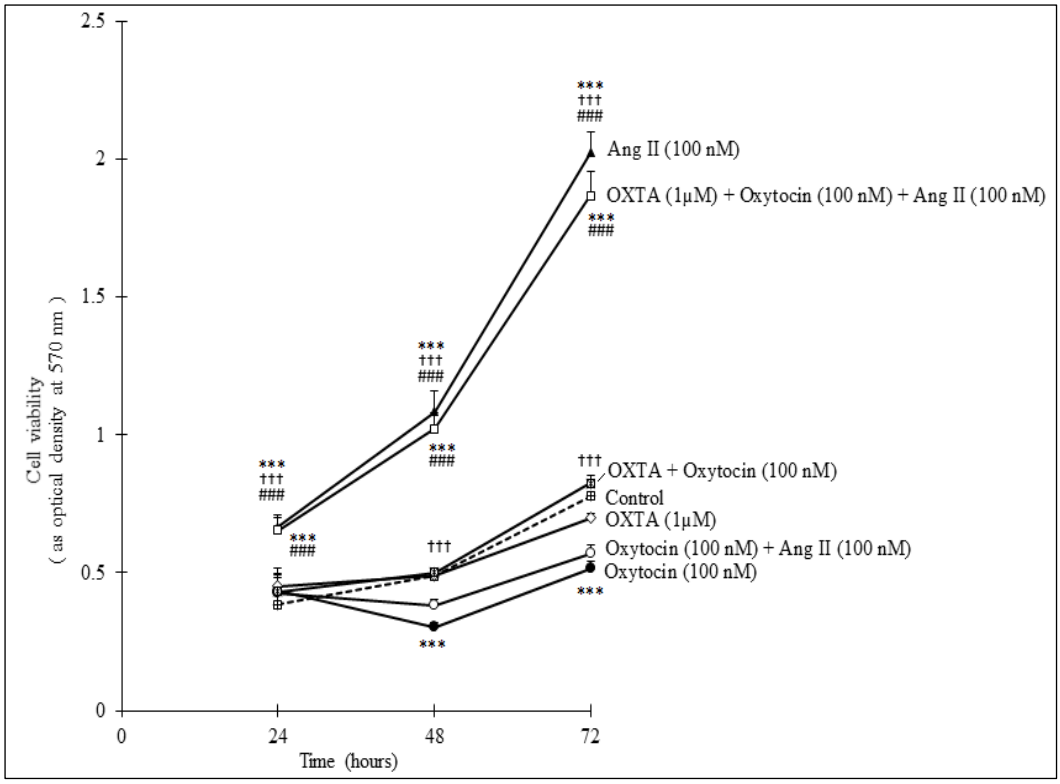

Figure 4.16. The effect of oxytocin and different treatment groups on aortic SHR VSMCs viability as assessed by the MTT assay. *SHR VSMCs were seeded at 1×10^4 cells/well in 96-well plates. Cell viability was determined as the function of an optical density (OD) measured at 570nm. The data are shown as the mean ± SEM of three independent experiments performed using aortic VSMCs isolated from three SHRs. Each experiment was performed with 12 replicates. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

*** *P < 0.001 : All groups versus Control*

††† *P < 0.001 : Oxytocin versus Ang II; and Oxytocin versus (OXTA + Oxytocin)*

P < 0.001 : (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

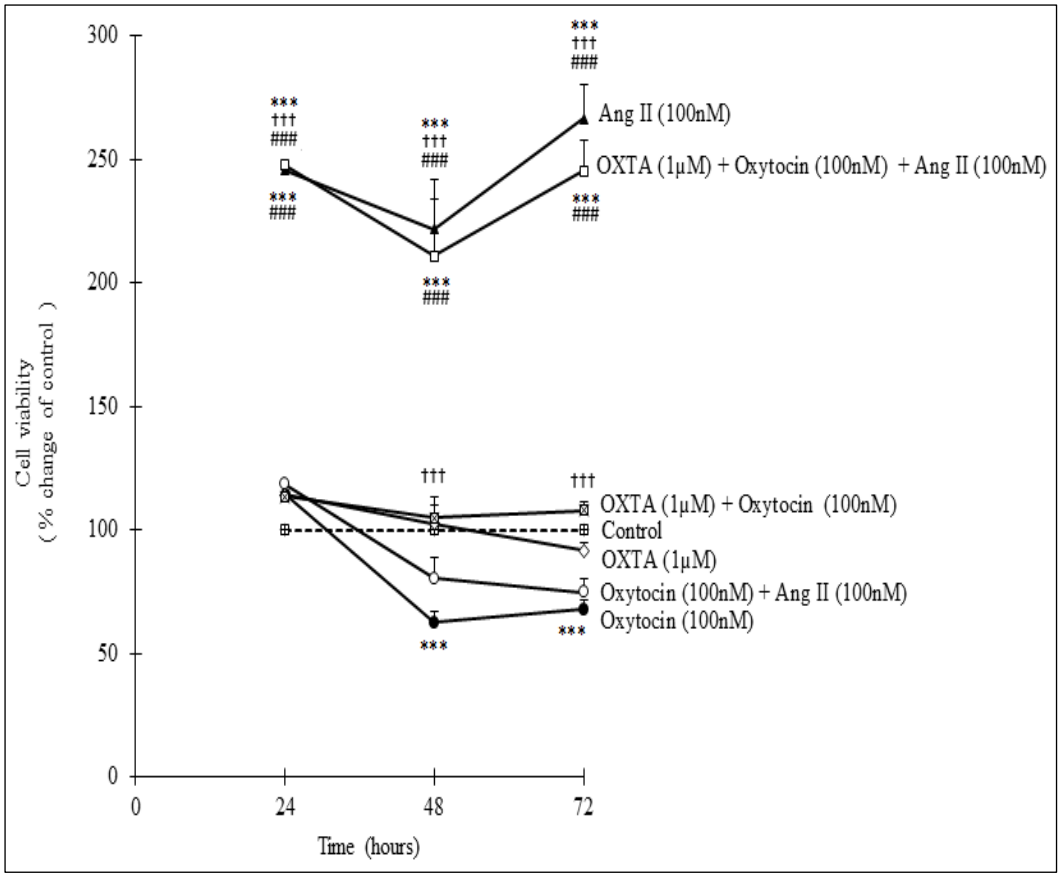

Figure 4.17. The effect of oxytocin and different treatment groups on aortic SHR VSMCs viability rate as assessed by the MTT assay. *SHR VSMCs were seeded at 1×10^4 cells/well in 96-well plates. Cell viability was measured as the percent (%) change of control. The data are shown as the mean ± SEM of three independent experiments performed using aortic VSMCs isolated from three SHRs. Each experiment was performed with 12 replicates. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

*** *P < 0.001 : All groups versus Control*

††† *P < 0.001 : Oxytocin versus Ang II; and Oxytocin versus (OXTA + Oxytocin)*

P < 0.001 : (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

4.2.1.3. Assessment of cell growth with ^{3}H-Thymidine incorporation assay in SHR VSMCs

The growth assay results corroborated that oxytocin (100 nM) significantly reduced ^{3}H-Thymidine incorporation in aortic SHR VSMCs from 2,668 ± 1,419 cpm (control) to 1,958 ± 2,473 cpm [36% ± 6% decrease ($P < 0.01$), **Figure 4.18**].

In contrast, treatment with Ang II (100 nM) significantly increased the ^{3}H-Thymidine incorporation into aortic SHR VSMCs from 2,668 ± 1,419 cpm (control) to 5,245 cpm ± 1,108 cpm [46% ± 71% increase ($P < 0.001$), **Figure 4.18**].

Pre-treatment with oxytocin (100 nM) followed by Ang II (100 nM) significantly decreased Ang II-induced growth of SHR VSMCs [1,385 cpm ± 1,170 cpm (73% ± 25% decrease), $P < 0.001$] compared to Ang II alone (5,245 ± 1,108 cpm), and these effects were antagonized by OXTA [1 μM (**Figure 4.18**)].

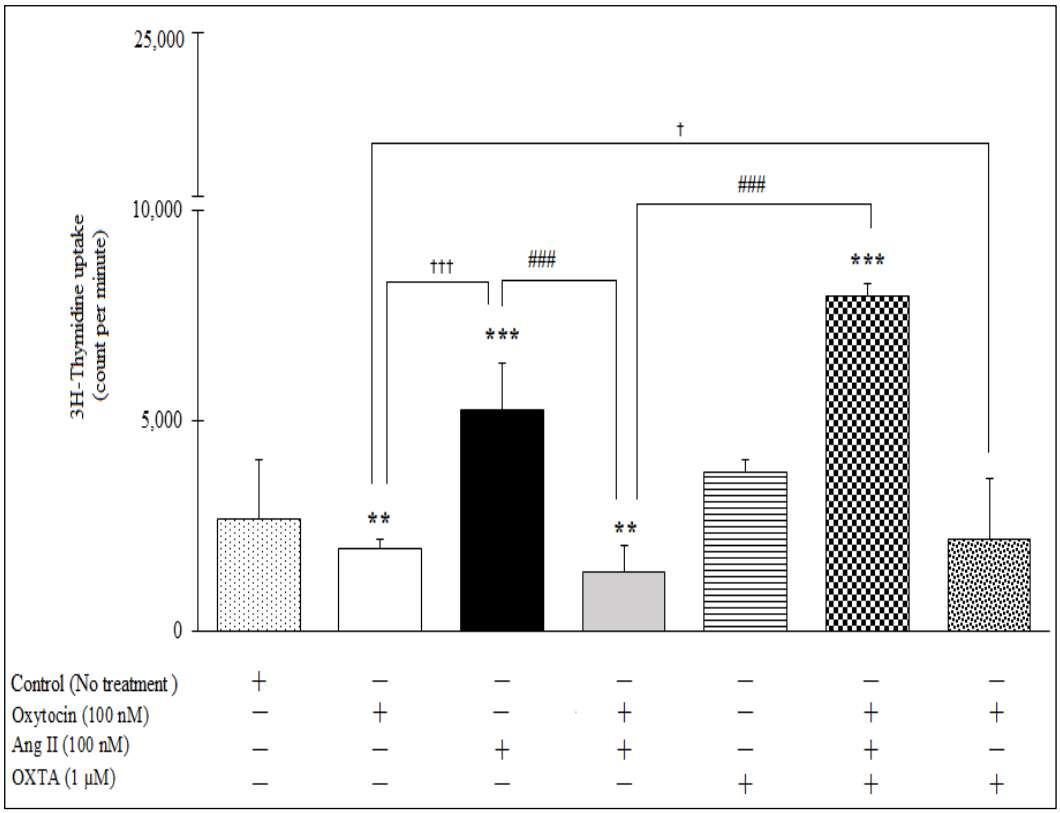

Figure 4.18. The effect of oxytocin and various treatments groups on aortic SHR VSMCs growth as assessed by the ^{3}H-Thymidine incorporation assay. *SHR VSMCs were seeded at 5×10^{4} cells/well in 24-well plates. Cell growth was measured as the function of the uptake of the radioisotope ^{3}H-Thymidine into the deoxyribonucleic acid (DNA) of the rapidly dividing cells and counted by the scintillation counter. The data are represented as the count per minute (cpm) and are shown as the mean ± SEM of four independent experiments, each performed with triplicates, using aortic VSMCs isolated from four SHRs. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

*** P < 0.01 and *** P < 0.001 : versus Control*

† P < 0.05 and ††† P < 0.001 : Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II*

P < 0.01 : (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II); and (Oxytocin + Ang II) versus Ang II alone*

4.2.1.4. Assessment of cell viability by flow cytometry in SHR VSMCs

In aortic SHR VSMCs, the flow cytometry results showed oxytocin treatment decreased the cell viability and induced apoptosis in a concentration-dependent manner, also caused necrosis (**Figure 4.19**).

The flow experiments utilized two different concentrations of oxytocin: 10 nM and 100 nM. When compared to the control group (93.4% ± 1.10%), the live cell percentage in aortic VSMCs significantly decreased to 80.9% ±0.7% ($P < 0.01$) in 10 nM oxytocin group and further declined to 65% ± 0.9% ($P < 0.01$) in 100 nM oxytocin group, respectively (**Figure 4.19**).

Oxytocin significantly induced apoptosis in aortic SHR VSMCs. The treatment with 10 nM and 100 nM oxytocin significantly increased apoptosis up to 13.9% ± 0.4% ($P < 0.001$) and 23.9% ± 0.1% ($P < 0.001$), respectively compared to apoptosis in the control group [2.6% ± 1.1% (**Figure 4.19**)].

In addition to inducing apoptosis, oxytocin considerably induced necrosis in aortic VSMCs. The percentage of aortic VSMCs undergoing necrosis significantly increased from 2.2% ± 0% (in control group) to 8.6% ± 1.3% ($P < 0.05$) in 100 nM oxytocin group. However, there was no significant difference between 10 nM oxytocin group (2.6% ± 0.3%) and the control group [2.2% ± 0% (**Figure 4.19**)].

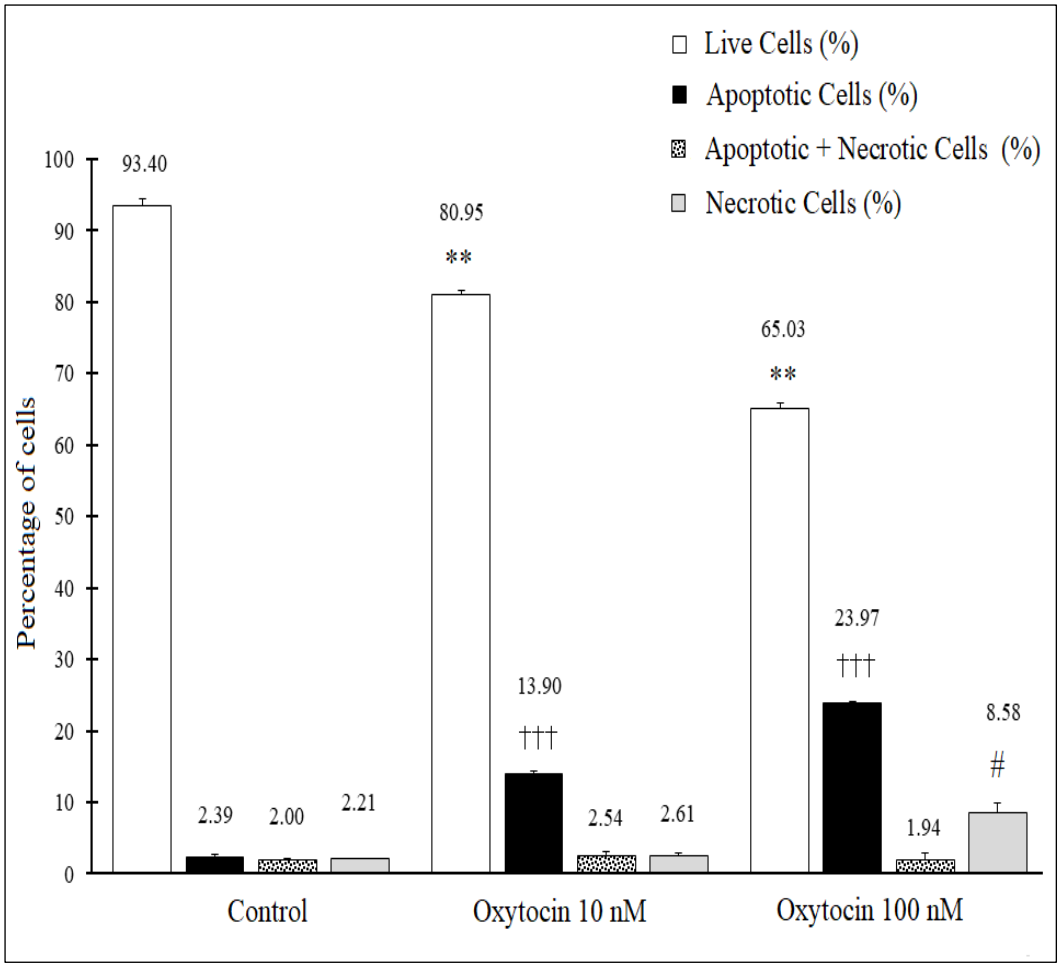

Figure 4.19. The effect of oxytocin on apoptosis and necrosis in aortic SHR VSMCs as assessed by flow cytometry. *SHR VSMCs were seeded at 1×10^6 cells/well in 6-well plates. The data are shown as the percentage of cells positive for apoptosis, necrosis and apoptosis + necrosis across different treatment groups. The data are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three SHRs. The treatment was carried out for 24 hours with the two different concentrations of oxytocin: 10 nM and 100 nM. The cells in the control group were not treated with oxytocin (only SFM). The cells were stained with fluorescent dyes: Annexin V (for apoptosis) and 7-amino actinomycin D [(7-AAD) for necrosis] and assessed with flow cytometer.*

*** P < 0.01 : Live cells in Oxytocin 10 nM group versus Live cells in Control group*

*** P < 0.01 Live cells in Oxytocin 100 nM group versus Live cells in Control group*

††† *P < 0.001 Apoptotic cells in Oxytocin 10 nM group versus Apoptotic cells in Control group*

††† *P < 0.001 Apoptotic cells in Oxytocin 100 nM group versus Apoptotic cells in Control group*

$^{\#}$ *P < 0.05 Necrotic cells in Oxytocin 100 nM group versus Necrotic cells in Control group.*

4.2.2. Effect of oxytocin and its pre-treatment with Ang II on the phosphorylation of ERK1/2 in aortic SHR VSMCs

In aortic VSMCs isolated and cultured from SHRs, the densitometric analysis of the western blot images showed that treatment with oxytocin (100 nM) significantly decreased the active phosphorylated form of ERK1/2 (pERK1/2) by 35.4% ± 4% ($P < 0.001$) compared to control (**Figure 4.20**).

Contrary to oxytocin's effect on pERK1/2 in SHR VSMCs, treatment with Ang II (100 nM) significantly increased phosphorylation of ERK1/2 by 157% ± 21% ($P < 0.001$) compared to control (**Figure 4.20**).

Interestingly, the oxytocin (100 nM) pre-treatment significantly reduced Ang II-induced pERK1/2 levels by 93.3% ± 1.4% ($P < 0.001$) compared to Ang II alone. The effects of oxytocin were significantly antagonized by the pre-treatment with 1 μM OXTA (**Figure 4.20**).

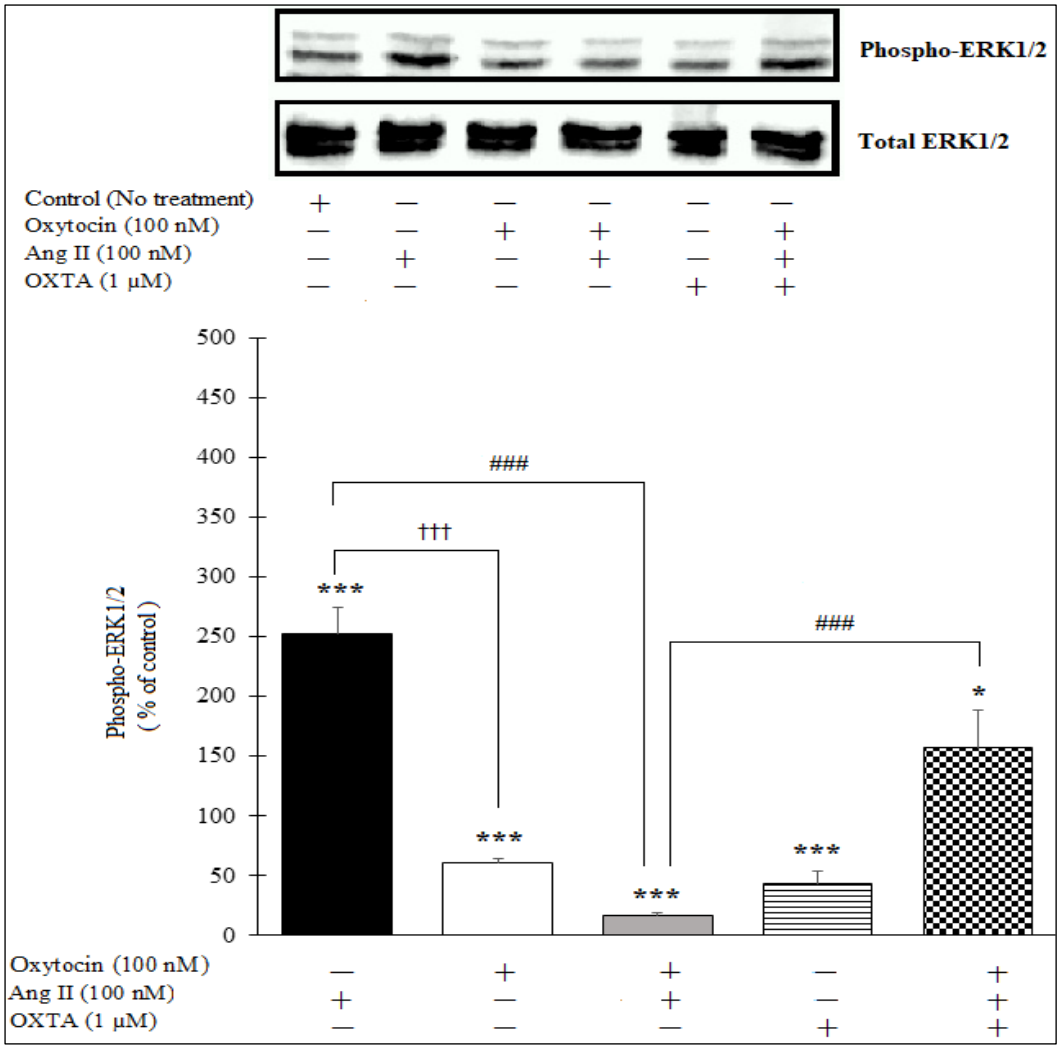

Figure 4.20. The effect of oxytocin and different treatment groups on the phosphorylation of ERK1/2 in aortic SHR VSMCs. *Western blotting was utilized to measure phospho-ERK1/2 levels, and the data were normalized with total ERK1/2. The effect of oxytocin and its combination with different treatment groups on the phosphorylation of ERK1/2 is shown as the percent (%) of control, with control as 100%. The data are represented as the mean ± SEM of five independent experiments, performed using aortic VSMCs isolated from five SHRs.* *** *indicates* $P < 0.001$ *versus control;* ††† $P < 0.001$ *Oxytocin versus Ang II;* ### $P < 0.001$ *(Oxytocin + Ang II) versus Ang II alone, and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

4.2.3. Effect of oxytocin and its pre-treatment with Ang II on the phosphorylation of PI3K p110α and Akt in aortic SHRs VSMCs

In aortic VSMCs cultured from SHRs, the densitometric analysis of western blot images showed that treatment with oxytocin (100 nM) significantly decreased the phosphorylated forms of PI3K p110α by 31% ± 5.1% ($P < 0.01$) and Akt by 45.5% ± 8.6% ($P < 0.01$) compared to control, respectively (**Figures 4.21** and **4.22**).

On the other hand, treatment with Ang II (100 nM) increased phosphorylated forms of PI3K p110α by 79.8% ± 10.3% ($P < 0.001$) and Akt by 70.2% ± 20.4% ($P < 0.05$) compared to control, respectively (**Figures 4.21** and **4.22**).

It is noteworthy that the oxytocin (100 nM) pre-treatment significantly reduced Ang II-induced higher levels of phospho-PI3K p110α by 61.3% ± 4.8% ($P < 0.01$) and phospho-Akt by 60.4% ± 2.6% ($P < 0.001$) compared to Ang II alone, respectively. And these effects of oxytocin were antagonized by pre-treatment with 1 μM OXTA (**Figures 4.21** and **4.22**).

Western blots depicting effect of oxytocin and various treatments on the phosphorylation of PI3K p110α and Akt in SHR VSMCs are shown in **Figures 4.21** and **4.22**, respectively.

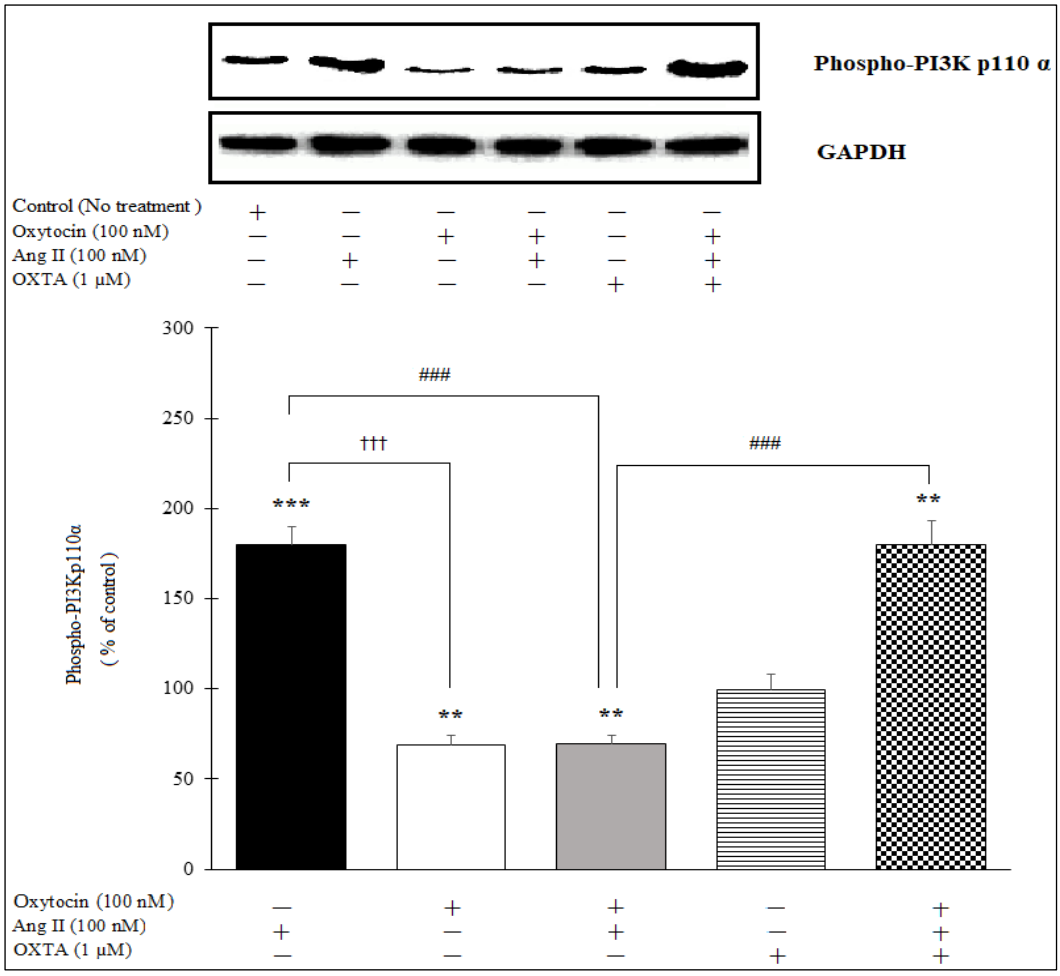

Figure 4.21. The effect of oxytocin and different treatment groups on the phosphorylation of PI3K p110α in aortic SHR VSMCs. *Western blotting was utilized to measure the phosphorylation of PI3K p110α, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the phosphorylation of PI3K p110α is shown as the percent (%) of control, with control as 100%. The data are shown as the mean ± SEM of three independent experiments, performed using aortic VSMCs isolated from three SHRs. ** indicates $P < 0.01$ versus control; *** $P < 0.001$ versus control; ††† $P < 0.001$ Oxytocin versus Ang II; ### $P < 0.001$ (Oxytocin + Ang II) versus Ang II alone, and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

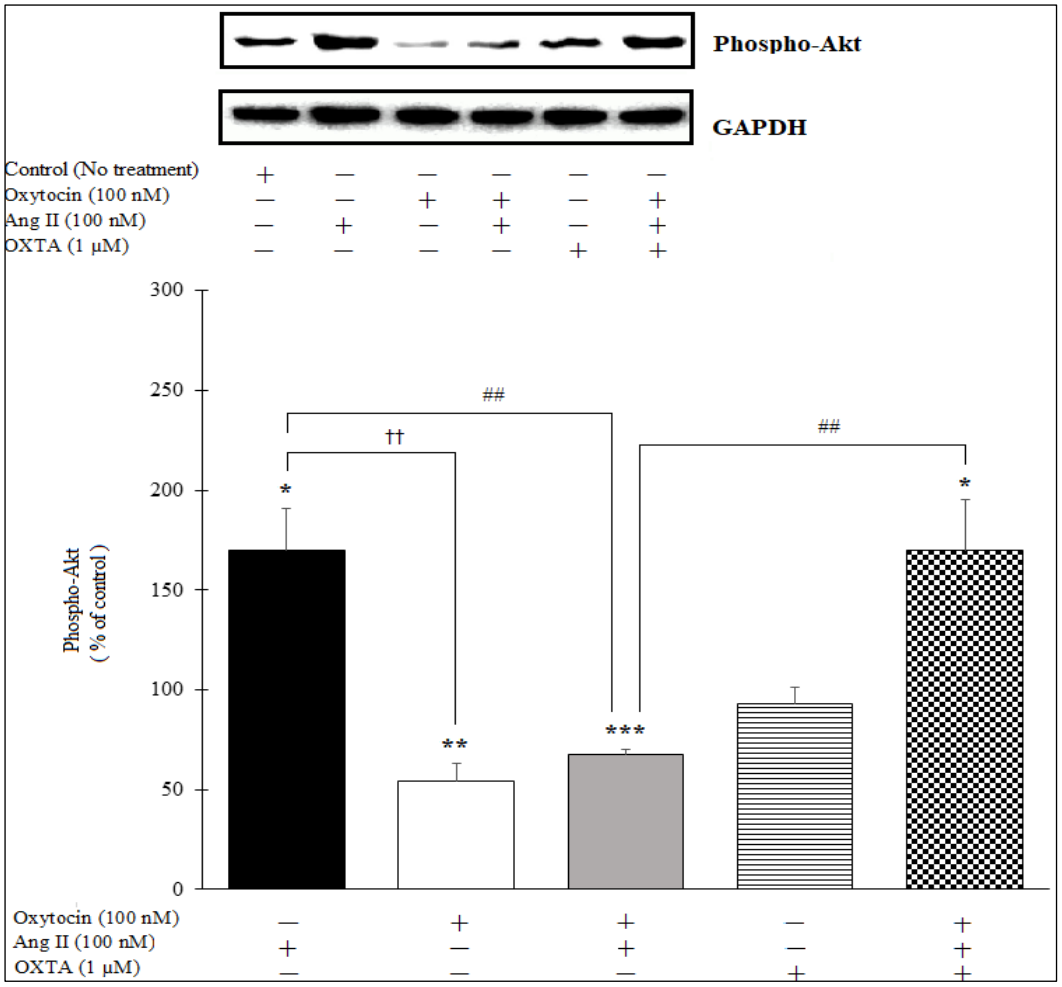

Figure 4.22. The effect of oxytocin and different treatment groups on the phosphorylation of Akt in aortic SHR VSMCs. *Western blotting was utilized to measure the phosphorylation of Akt, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the phosphorylation of Akt is shown as the percent (%) of control, with control as 100%. The data are shown as the mean ± SEM of four independent experiments, performed using aortic VSMCs isolated from four SHRs. * indicates $P < 0.05$ versus control; ** $P < 0.01$ versus control; *** $P < 0.001$ versus control; $^{††}P < 0.01$ Oxytocin versus Ang II; $^{\#\#}P < 0.01$ (Oxytocin + Ang II) versus Ang II alone, and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

4.2.4. Effect of oxytocin and its pre-treatment with Ang II on the activation of ROCK -1 and ROCK-2 in aortic SHR VSMCs

In aortic SHR VSMCs, the densitometric analysis of western blot images showed treatment with oxytocin (100 nM) significantly reduced the activation of ROCK-1 by 58.9% ± 5.4% ($P < 0.001$) and ROCK-2 by 41.5% ± 12.8% ($P < 0.01$) compared to control, respectively (**Figures 4.23** and **4.24**).

Treatment with Ang II (100 nM) prominently increased the activation of ROCK-1 by 102% ± 43% ($P < 0.05$) and ROCK-2 by 81.2% ± 14.8% ($P < 0.001$) compared to control, respectively (**Figures 4.23** and **4.24**).

Pre-treatment with oxytocin (100 nM) significantly reduced Ang II-induced activation of ROCK-1 by 79% ± 6.9% ($P < 0.01$) and ROCK-2 by 69.5% ± 13.2% ($P < 0.001$) compared to Ang II alone, respectively. These effects of oxytocin were significantly blocked by pre-treatment with 1μM OXTA (**Figures 4.23** and **4.24**).

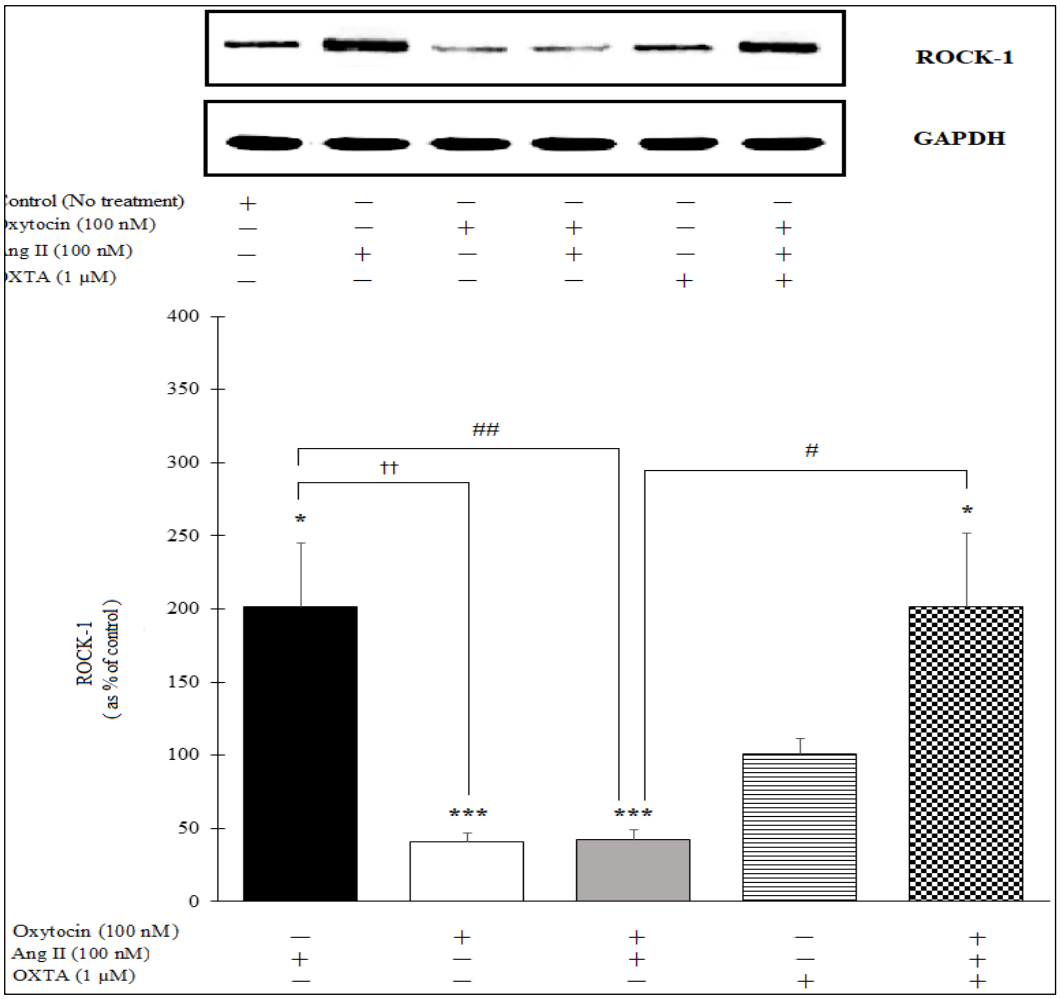

Figure 4.23. The effect of oxytocin and different treatment groups on the activation of ROCK-1 in aortic SHR VSMCs. *Western blotting was utilized to measure the ROCK-1 levels, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the activation of ROCK-1 is shown as the percent (%) of control, with control as 100%. The data are shown as the mean ± SEM of four independent experiments, performed using aortic VSMCs isolated from four SHRs. * indicates $P < 0.05$ versus control; *** $P < 0.001$ versus control; †† $P < 0.01$ Oxytocin versus Ang II; ## $P < 0.01$ Oxytocin + Ang II versus Ang II alone; # $P < 0.05$ Oxytocin + Ang II versus OXTA + Oxytocin + Ang II. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist; ROCK, Rho-associated coiled-coil-containing protein kinase.*

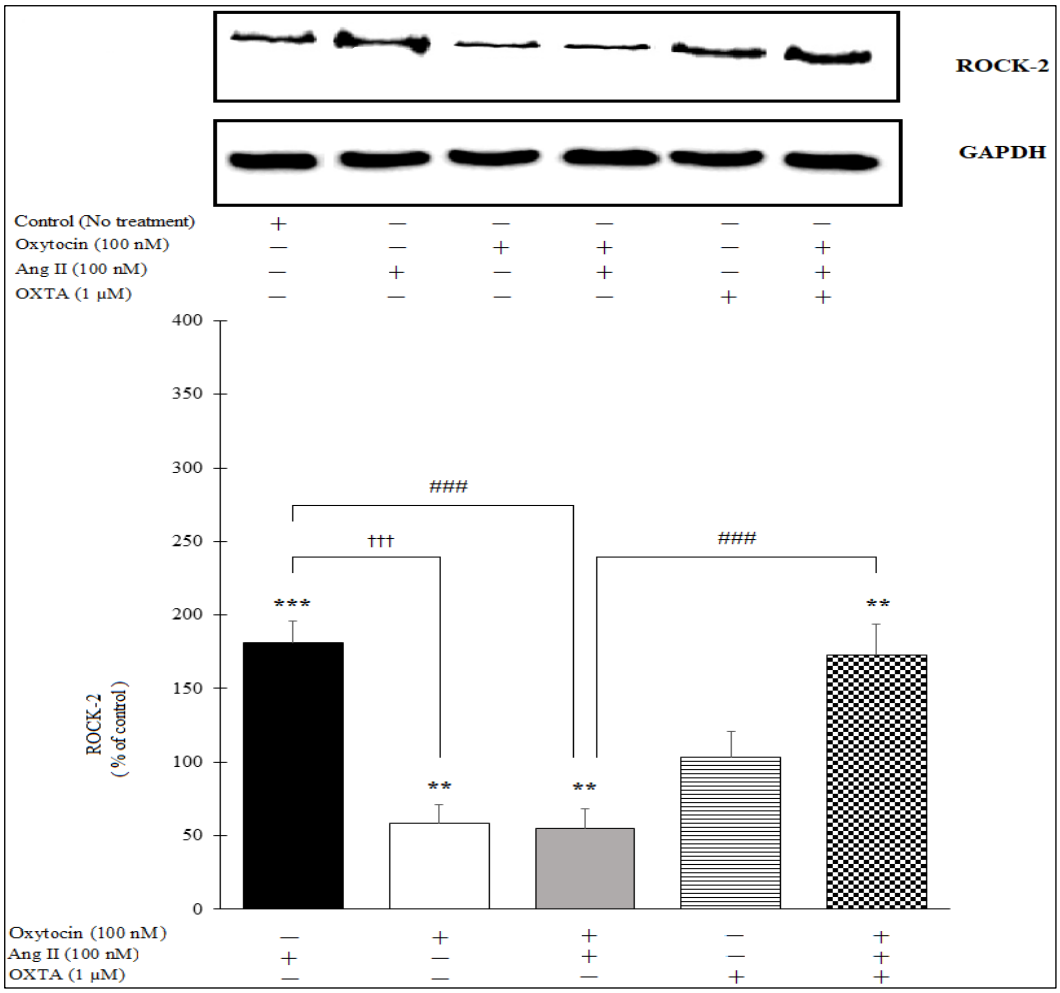

Figure 4.24. The effect of oxytocin and different treatment groups on the activation of ROCK-2 in aortic SHR VSMCs. *Western blotting was utilized to measure the ROCK-2 levels, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the activation of ROCK-2 as the percent (%) of control, with control as 100%. The data are shown as the mean ± SEM of five independent experiments, performed using aortic VSMCs isolated from five SHRs. ** indicates $P < 0.01$ versus control; *** $P < 0.001$ versus control; ††† $P < 0.01$ Oxytocin versus Ang II; ### $P < 0.01$ Oxytocin + Ang II versus Ang II alone, and Oxytocin + Ang II versus OXTA + Oxytocin + Ang II. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist; ROCK, Rho-associated coiled-coil containing protein kinase.*

4.2.5. Effect of oxytocin and its pre-treatment with Ang II on IL-6 secretion in aortic SHR VSMCs

As per the manufacturer's protocol, the IL-6ELISA assay was carried out and the concentration of IL-6 secreted by the aortic VSMCs in the culture media was measured as 'picogram per milliliter' (pg/mL). In spontaneously hypertensive rats (SHRs), the findings of the IL-6 assay demonstrated that oxytocin significantly increased IL-6 secretion in culture media compared to the control group (**Figure 4.25)**.

The assay employed three different concentrations of oxytocin: 10 nM, 100 nM, 1,000 nM. When compared with control group (848 ± 9 pg/mL), oxytocin significantly increased IL-6 secretion in a concentration-dependent manner: 985 ± 25 pg/mL ($P < 0.01$) at 10 nM; 1,289 ± 8 pg/mL ($P < 0.001$) at 100 nM; and 1,468 ± 19 pg/mL ($P < 0.01$) at 1,000 nM, respectively (**Figure 4.25**).

The treatment with Ang II (100 nM) showed a significant increase in IL-6 secretion [1,620 ± 66 pg/mL ($P < 0.001$)] compared to the control group [(848 ± 9 pg/mL), **Figure 4.25**].

When oxytocin (100 nM) and Ang II (100 nM) groups were compared to the control group, Ang II (100 nM) showed significantly more IL-6 [1,620 ± 66 pg/mL ($P < 0.01$)] than oxytocin [100 nM (1,289 ± 8 pg/mL)] compared to the control group [(848 ± 9 pg/mL), **Figure 4.25**].

Although oxytocin on its own significantly increased IL-6 secretion, interestingly, oxytocin's pre-treatment (100 nM) with Ang II (100 nM) significantly decreased IL-6 secretion [891 ± 59 pg/mL ($P < 0.001$)] compared to

Ang II 100 nM alone (1,620 ± 66 pg/mL) and oxytocin 100 nM alone [1,289 ± 8 pg/mL ($P < 0.001$)], respectively (**Figure 4.25**).

The effect of oxytocin's pre-treatment (100 nM) on Ang II (100 nM) was significantly inhibited by pre-treatment with 1μM OXTA. IL-6 secretion was significantly higher [1,476 ± 58 pg/mL ($P < 0.01$)] in the OXTA-pre-treatment group [OXTA (1μM) + Oxytocin (100 nM) + Ang II (100 nM)] than Oxytocin 100 nM + Ang II 100 nM group [(891 ± 59 pg/mL), **Figure 4.25**].

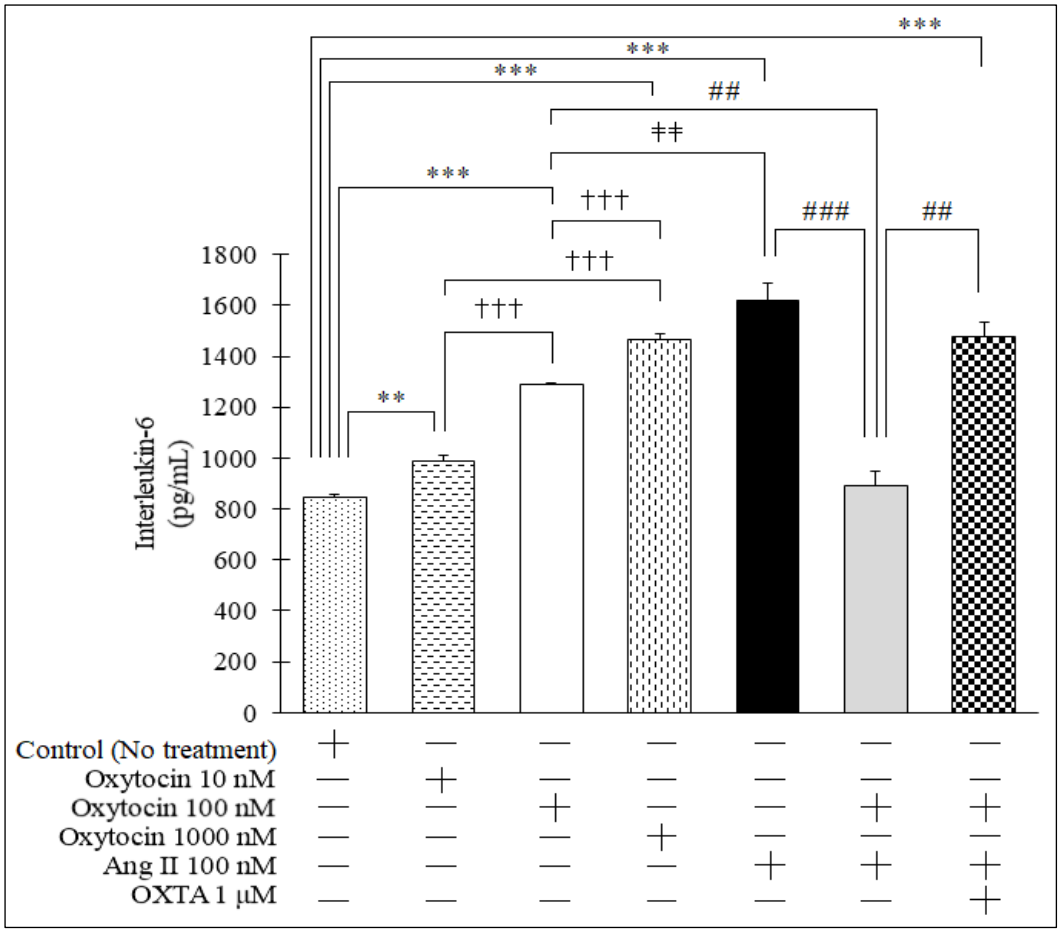

Figure 4.25. The effect of oxytocin and different treatment groups on IL-6 secretion in aortic SHR VSMCs. *The concentration of IL-6 secreted by aortic SHR VSMCs in culture media was measured in picogram per mL (pg/mL). The data are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three SHRs. In each experiment each treatment group had duplicates. ** indicates P < 0.01 versus Control group; *** P < 0.001 versus Control group; ††† P < 0.001 Oxytocin 10 nM versus Oxytocin 100 nM and Oxytocin 1000 nM respectively, and Oxytocin 100 nM versus Oxytocin 1000 nM. ## P < 0.01 Oxytocin 100 nM + Ang II 100 nM versus Oxytocin 100 nM alone and OXTA 1µM + Oxytocin 100 nM + Ang II 100 nM; ### P < 0.001 Oxytocin 100 nM + Ang II 100 nM versus Ang II 100 nM alone; ‡‡ P < 0.01 Oxytocin 100 nM versus Ang II 100 nM. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

4.2.6. Effect of oxytocin and its pre-treatment with Ang II on ROS production in aortic SHR VSMCs

The outcomes of the ROS assay in aortic SHR VSMCs revealed that oxytocin significantly increased ROS production and its pre-treatment with Ang II also considerably increased ROS production (**Figure 4.26**).

Measured as the function of fluorescent units, the ROS assay findings showed that treatment with oxytocin (100 nM) significantly increased ROS production [6,657 ± 202 ($P < 0.001$)] compared to the control group (5,411 ± 259) in aortic SHR VSMCs. The treatment with Ang II (100 nM) significantly increased ROS production [6,286 ± 208 ($P < 0.001$)] compared to the control group (5,411 ± 259) (**Figure 4.26**).

There was no significant difference in ROS production between oxytocin [100 nM (6,657 ± 202)] and Ang II [100 nM (6,286 ± 208)] treatment groups (**Figure 4.26**).

The pre-treatment of oxytocin (100 nM) followed by Ang II (100 nM) significantly increased ROS production [7,481 ± 162 ($P < 0.001$)] compared to the control group [5,411 ± 259 (**Figure 4.26**)].

Additionally, pre-treatment with Oxytocin (100 nM) followed by Ang II (100 nM) significantly increased the ROS production (7,481 ± 162) compared to oxytocin (100 nM) alone [6,657 ± 202 ($P < 0.01$)] and Ang II (100 nM) alone [6,286 ± 208 ($P < 0.001$)], respectively, indicating an additive effect (**Figure 4.26**).

Interestingly, OXTA (1 μM) on its own significantly increased the ROS production [6,703 ± 173 ($P < 0.001$)] compared to the control group (5,411 ± 259).

In addition, pre-treatment with OXTA (1 μM) followed by Oxytocin (100 nM) also significantly increased ROS production [6,978 ± 223 ($P < 0.001$)] compared to control group [5,411 ± 259 (**Figure 4.26**)].

There was no significant difference between Oxytocin (100 nM) group (6,657 ± 202) and OXTA (1 μM) + Oxytocin (100 nM) group [6,978 ± 223 (**Figure 4.26**)].

The combination of OXTA (1 μM), Oxytocin (100 nM), and Ang II significantly increased ROS production [7,705 ± 211 ($P < 0.001$)] compared to control (5,411 ± 259) in aortic SHR VSMCs (**Figure 4.26**).

The combination of OXTA, Oxytocin, and Ang II (100 nM) significantly increased ROS production [7,705 ± 211 ($P < 0.001$)] compared to Oxytocin (100 nM) alone (6,657 ± 202), Ang II (100 nM) alone (6,286 ± 208) and OXTA (1 μM) alone (6,703 ± 173), respectively, indicating an additive effect (**Figure 4.26**).

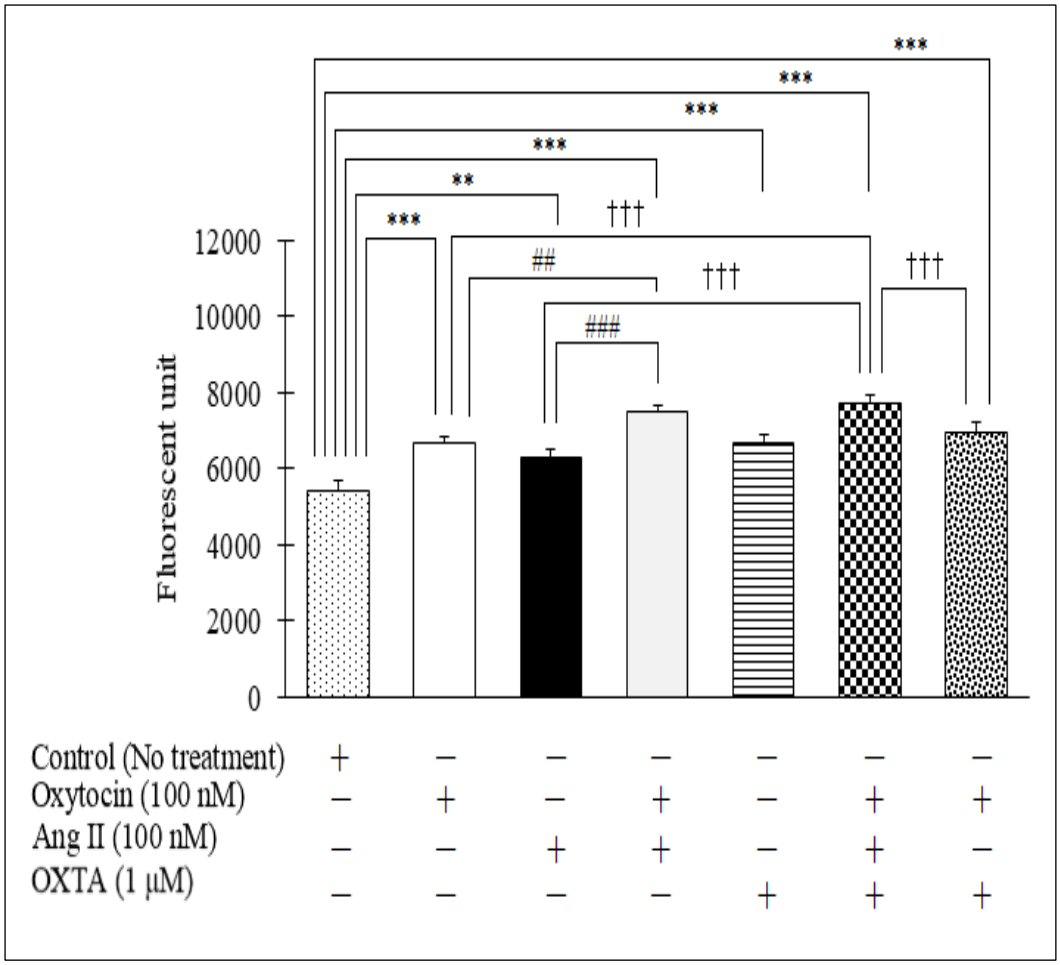

Figure 4.26. The effect of oxytocin and different treatment groups on ROS production in aortic SHR VSMCs. *SHR VSMCs were seeded at 1×10^4 cells/well in 96-well plates. The ROS levels were measured as the function of the fluorescent unit. The data are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three SHRs. In each experiment each treatment group had 12 replicates. ** indicates P < 0.01 Ang II versus Control; *** P < 0.001 versus Control group; $^{\#\#}$ P < 0.01 Oxytocin + Ang II versus Oxytocin alone; $^{\#\#\#}$ Oxytocin + Ang II versus Ang II alone; †††P < 0.001 OXTA + Oxytocin + Ang II versus Oxytocin alone, Ang II alone, and OXTA alone. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

4.3. Comparison of the effects of oxytocin and its pre-treatment with Ang II on aortic Wistar VSMCs versus SHR VSMCs

The effects of oxytocin on aortic Wistar VSMCs and SHR VSMCs alone and after pre-treatment with Ang II were analyzed and compared, and are explained under the following sub-headings:

4.3.1. Comparison between Wistar VSMCs and SHR VSMCs regarding the effect of oxytocin and its pre-treatment with Ang II on aortic VSMCs proliferation

In control group, the proliferation of Wistar VSMCs was significantly higher at 24 hours [6.37×10^6 cells/mL ± 9.20×10^5 cells/mL ($P < 0.05$)] and 48 hours [1.50×10^7 cells/mL ± 1.47×10^6 cells/mL ($P < 0.05$)] compared to SHR VSMCs (4.24×10^6 cells/mL ± 1.51×10^5 cells/mL at 24 hours and 1.10×10^7 cells/mL ± 7.04×10^5 at 48 hours). However, at 72 hours there was no significant difference in proliferation between Wistar VSMCs (2.30×10^7 cells/mL ± 5.53×10^6 cells/mL) and SHR VSMCs [1.59×10^7 cells/mL 2.72×10^6 cells/mL (**Figure 4.27**)].

Oxytocin significantly decreased proliferation in Wistar VSMCs: 3.12×10^6 cells/mL ± 4.14×10^5 cells/mL at 24 hours; 1.64×10^6 cells/mL ± 3.07×10^5 cells/mL at 48 hours; and 1.23×10^6 cells/mL ± 4.45×10^5 cells/mL at 72 hours post-treatment compared to their respective control. Similar to the results in Wistar VSMCs, oxytocin remarkably decreased proliferation in SHR VSMCs: 2.34×10^6 cells/mL ± 1.98×10^5 cells/mL at 24 hours; 1.72×10^6 cells/mL ± 1.04×10^5 cells/mL at 48 hours; and 1.71×10^6 cells/mL ± 4.29×10^5 cells/mL at 72 hours

post-treatment compared to their respective control. It is noteworthy that there was no significant difference in the pattern and magnitude of oxytocin-induced inhibition of proliferation in Wistar VSMCs and SHR VSMCs (**Figure 4.27**).

Ang II showed significantly more proliferation in Wistar VSMCs [2.88×10^7 cells/mL ± 1.74×10^6 cells/mL ($P < 0.001$) at 24 hours; 4.73×10^7 cells/mL ± 3.45×10^6 cells/mL ($P < 0.01$) at 48 hours; and 5.03×10^7 cells/mL ± 1.82×10^6 cells/mL ($P < 0.05$) at 72 hours post-treatment] than SHR VSMCs (1.22×10^7 cells/mL ± 2.98×10^5 cells/mL at 24 hours; 2.33×10^7 cells/mL ± 1.03×10^6 cells/mL at 48 hours; and 4.88×10^7 cells/mL ± 9.99×10^5 cells/mL at 72 hours post-treatment) compared to their respective controls (**Figure 4.27**).

Oxytocin pre-treatment with Ang II significantly reduced Ang II-mediated proliferation of VSMCs isolated from both Wistar rats (1.07×10^7 cells/mL ± 2.99×10^6 cells/mL at 24 hours; 8.06×10^6 cells/mL ± 3.13×10^6 cells/mL at 48 hours; and 6.55×10^6 cells/mL ± 2.23×10^6 cells/mL at 72 hours post-treatment) and SHRs (4.14×10^6 cells/mL ± 8.47×10^5 cells/mL at 24 hours; 1.60×10^6 cells/mL ± 2.80×10^5 cells/mL at 48 hours; and 1.37×10^6 cells/mL ± 2.32×10^5 cells/mL at 72 hours post-treatment) compared to their respective Ang II (alone) groups. However, this effect was significantly higher in SHRs than Wistar Rats at 24 hours ($P < 0.05$), 48 hours ($P < 0.05$), and 72 hours ($P < 0.05$) post-treatment (**Figure 4.27**).

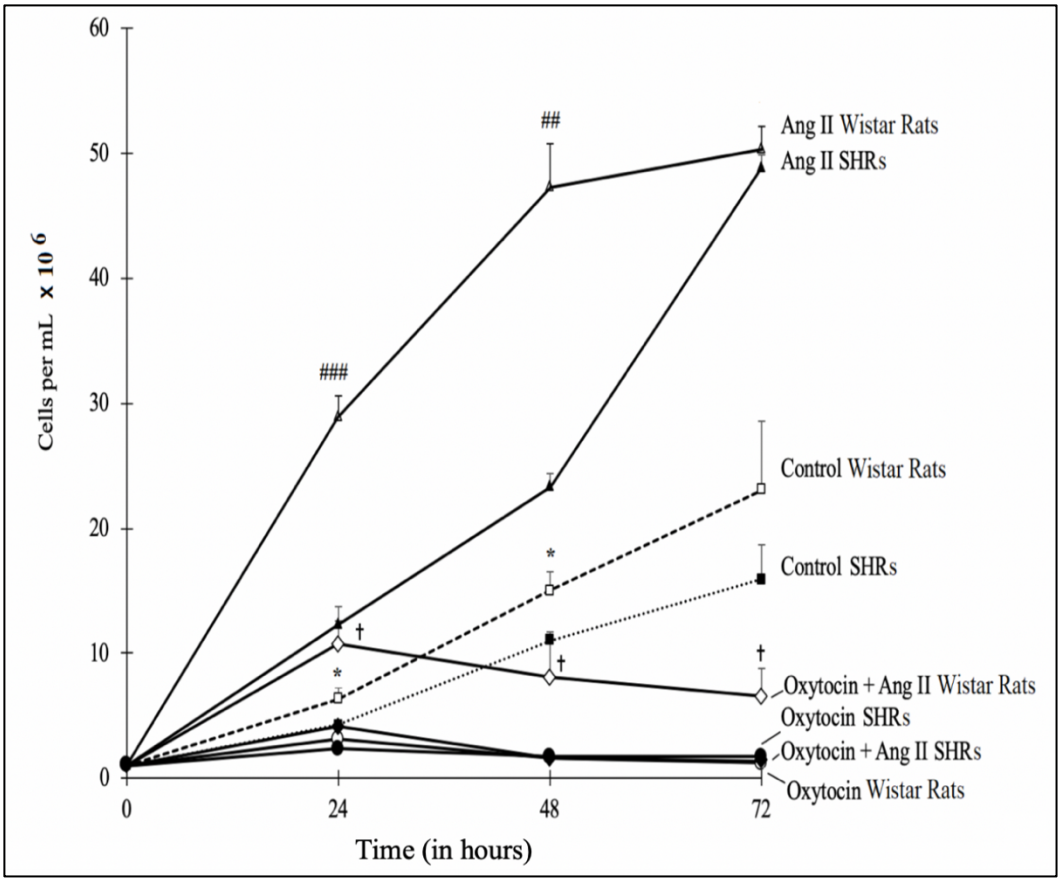

Figure 4.27. The comparison of the effects of oxytocin, Ang II, and the pre-treatment of oxytocin with Ang II on proliferation of aortic VSMCs between Wistar VSMCs and SHR VSMCs as assessed by the Hemocytometer method. *VSMCs isolated from both Wistar rats and SHRs were seeded at 1×10^6 cells/well in 6-well plates. Cell proliferation was measured by counting the number of cells per milliliter (mL). The data are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three Wistar rats and three SHRs, respectively.* * *indicates $P < 0.05$ versus Control Wistar rats versus Control SHRs;* † *$P < 0.05$ (Oxytocin + Ang II) Wistar rats versus (Oxytocin + Ang II) SHRs;* ## *$P < 0.01$ Ang II Wistar rats vs Ang II SHRs;* ### *$P < 0.001$ Ang II Wistar rats vs Ang II SHRs. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

4.3.2. Comparison between Wistar VSMCs and SHR VSMCs regarding the effect of oxytocin and its pre-treatment with Ang II on aortic VSMCs viability

In MTT assay, the cell viability was determined as the function of the optical density (OD) measured at 570 nm. On comparing the cell viability of aortic VSMCs isolated from both Wistar rats and SHRs, significantly higher cell viability was observed in the control group SHR VSMCs [0.4 OD ± 0.1 ($P < 0.05$)] OD at 24 hours than Wistar VSMCs (0.3 OD ± 0.0 OD). However, at 48 hours and 72 hours there was no significant difference in the cell viability between Wistar VSMCs (0.5 OD ± 0.0 OD at 48 hours and 0.7 OD ± 0.0 OD and 72 hours) and SHR VSMCs [0.5 OD ± 0.0 OD at 48 hours and 0.8 OD ± 0.0 OD at 72 hours (**Figure 4.28**).

Oxytocin significantly decreased the cell viability of aortic VSMCs isolated from both Wistar rats and SHRs compared to the control groups, respectively. At 24 hours post-treatment, Oxytocin significantly decreased more cell viability of Wistar VSMCs [0.2 OD ± 0.0 OD ($P < 0.05$)] than SHR VSMCs (0.4 OD ± 0.1 OD). However, at 48 hours and 72 hours there was no significant difference in the oxytocin-induced decrement in viability between the Wistar VSMCs (0.3 OD ± 0.0 OD at 48 hours and 0.6 OD ± 0.1 OD at 72 hours) and SHR VSMCs (0.3 OD ± 0.0 OD at 48 hours and 0.5 OD ± 0.0 OD at 72 hours). Oxytocin showed a similar pattern and magnitude of inhibition in cell viability in both Wistar VSMCs and SHR VSMCs at 48 hours and 72 hours post-treatment (**Figure 4.28**).

Ang II significantly increased the cell viability of aortic VSMCs isolated from both Wistar rats (0.5 OD ± 0.1 OD at 24 hours, 1.0 OD ± 0.1 OD at 48 hours, and 1.3 OD ± 0.1 OD at 72 hours) and SHRs (0.7 OD ± 0.0 OD at 24 hours, 1.1 OD ± 0.1 OD at 48 hours, and 2.0 OD ± 0.1 OD at 72 hours) compared to the respective control groups. However, at 72 hours post-treatment, Ang II showed a more significant increase in cell viability in SHR VSMCs [2.0 OD ± 0.1 OD ($P < 0.001$)] than Wistar VSMCs [1.3 OD ± 0.1 OD (**Figure 4.28**)].

The pre-treatment of oxytocin with Ang II significantly decreased Ang II-induced cell viability in both Wistar VSMCs (0.2 OD ± 0.0 OD at 24 hours, 0.3 OD ± 0.0 OD at 48 hours, and 0.6 OD ± 0.1 OD at 72 hours) and SHR VSMCs (0.4 OD ± 0.1 OD at 24 hours, 0.3 OD ± 0.0 OD at 48 hours, and 0.6 OD ± 0.0 OD at 72 hours) compared to the respective Ang II (alone) groups (**Figure 4.28**).

At 24 hours post-treatment, the oxytocin-pretreatment showed more significant decrease viability in Wistar VSMCs [0.2 OD ± 0.0 OD ($P < 0.01$)] than SHR VSMCs (0.4 OD ± 0.1 OD). Although, at 48 and 72 hours, respectively, there was no significant difference in the cell viability between Wistar VSMCs and SHR VSMCs in the oxytocin-pre-treatment with Ang II group (**Figure 4.28**).

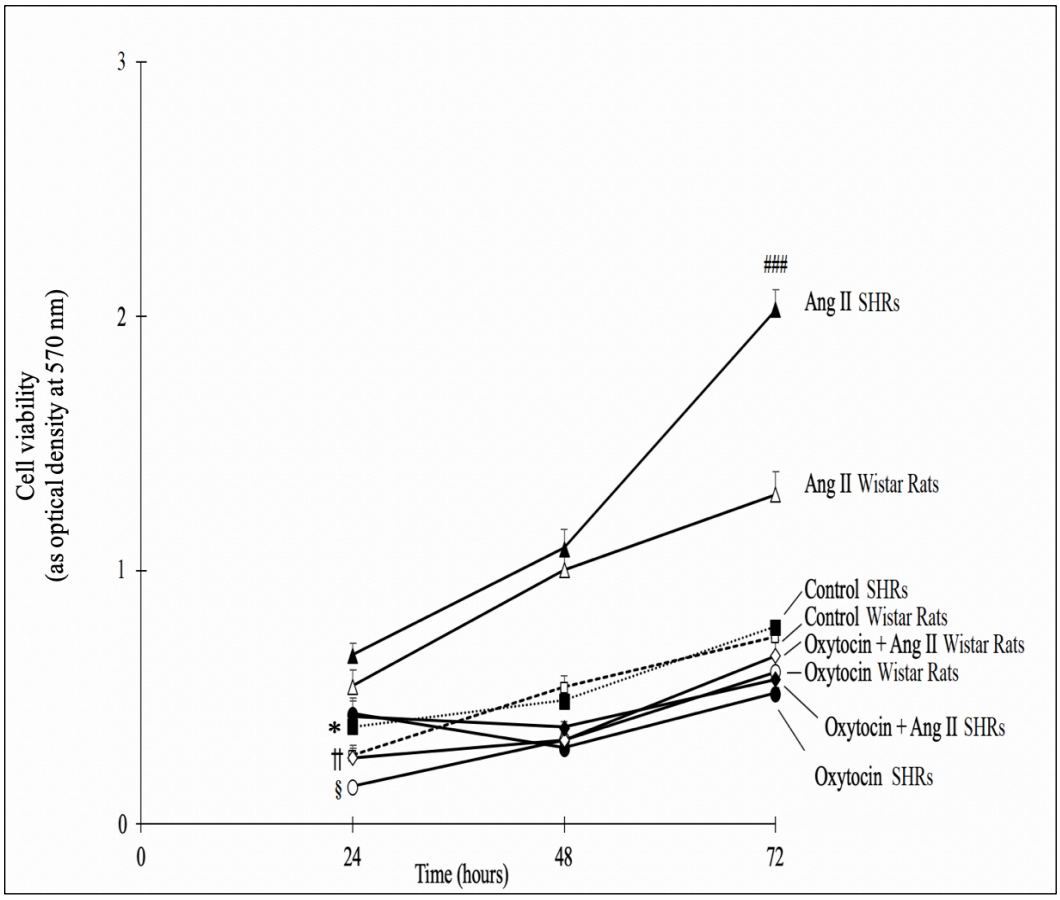

Figure 4.28. The comparison of the effects of oxytocin, Ang II, and the pre-treatment of oxytocin with Ang II on viability of aortic Wistar VSMCs and SHR VSMCs as assessed by the MTT assay. *VSMCs isolated from both Wistar rats and SHRs were seeded at 1×10^4 cells/well in 96-well plates. Cell viability was measured as the function of an optical density (OD) at 570 nm. The data are shown as the mean ± SEM from three independent experiments performed using aortic VSMCs from three Wistar rats and three SHRs, respectively. Each experiment was performed with 12 replicates. * indicates $P < 0.05$ Control Wistar rats versus Control SHRs; § $P < 0.05$ Oxytocin Wistar rats versus Oxytocin SHRs;* †† $P < 0.01$ (*Oxytocin + Ang II) Wistar rats versus (Oxytocin + Ang II) SHRs;* $^{\#\#\#}$ *$P < 0.001$ Ang II Wistar rats versus Ang II SHRs. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.*

4.3.3. Comparison of the effect of oxytocin on apoptosis and necrosis between Wistar VSMCs and SHR VSMCs

In aortic VSMCs isolated from both Wistar rats and SHRs, the flow cytometry results showed that oxytocin significantly reduced live cells percentage, prominently induced apoptosis (in a concentration-dependent manner) and induced necrosis.

The flow experiments employed two different concentrations of oxytocin: 10 nM and 100 nM. In the control group, there was no significant difference between: live cells in Wistar VSMCs (95.5 ± 0.7%) and SHR VSMCs (93.4% ± 1.1% in SHRs); and apoptotic cells in Wistar VSMCs (2.7% ± 1.1%) and SHR VSMCs (2.4% ± 0.3%). However, the SHR VSMCs control group showed a significantly higher percentage of necrotic cells [1.5% ± 0.0% ($P < 0.01$)] compared to the Wistar VSMCs control group [0.7% ± 0.1% (**Figure 4.29**)].

The treatment with oxytocin 10 nM resulted in a more significant reduction in live cells percentage in SHR VSMCs [80.9% ± 0.7% ($P < 0.01$)] than Wistar VSMCs (91.6% ± 0.4%) compared to their respective controls. In addition, oxytocin 10 nM induced significantly more apoptosis in SHR VSMCs [13.9% ± 0.4% ($P < 0.05$)] than Wistar VSMCs (4.2% ± 1.6%) compared to their respective controls (**Figure 4.29**).

Treatment with oxytocin 100 nM showed a similar pattern and magnitude in reduction of the percentage of live cells and induction of apoptosis in between Wistar VSMCs (67.5% ± 2.3% live cells and 26.1% ± 3.0% apoptotic cells) and SHR VSMCs (65.0% ± 0.9% live cells and 23.9% ± 0.1% apoptotic cells) compared

to their respective controls. Interestingly, oxytocin 100 nM resulted in a significantly higher percentage of cells undergoing necrosis [8.6% ± 1.3% ($P < 0.05$)] in SHR VSMCs than Wistar VSMCs [3.9% ± 0.3% (**Figure 4.29**)].

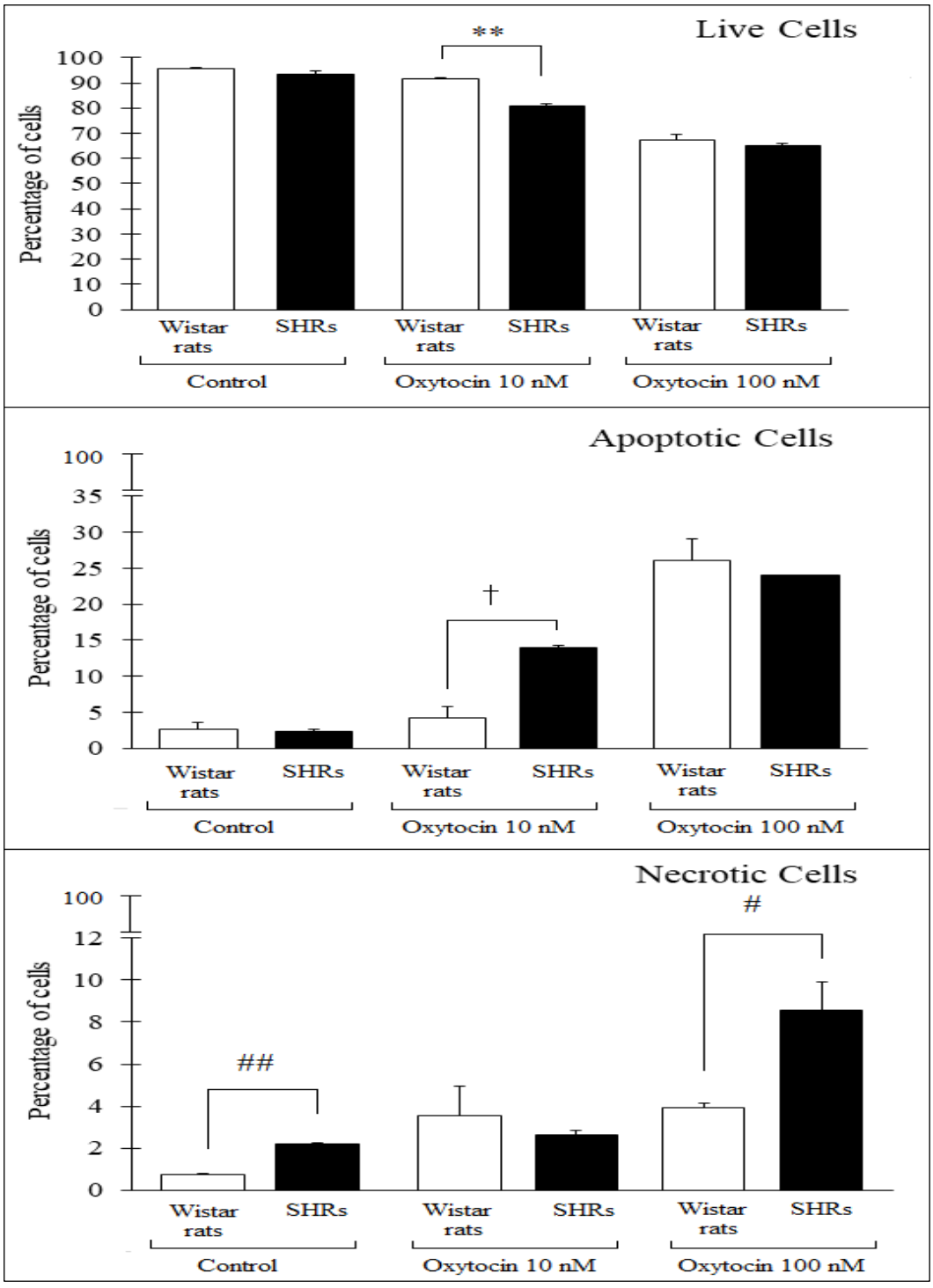

Figure 4.29. The comparison of the effects of oxytocin on the live cells, apoptosis, and necrosis between Wistar VSMCs and SHR VSMCs as assessed by flow cytometry. *Aortic VSMCs isolated from both Wistar rats and SHRs were seeded at 1×10^6 cells/well in 6-well plates. The data are shown as the percentage of cells and*

are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three Wistar rats and three SHRs, respectively. The treatment was carried out 24 hours with two different concentrations of oxytocin: 10nM and 100nM. The cells were stained with fluorescent dyes: Annexin V (for apoptosis) and 7-amino actinomycin D [(7-ADD) for necrosis]. ** *indicates P < 0.01 Live Cells in Oxytocin 10 nM group in Wistar rats versus Live Cells in Oxytocin 100 nM in group SHRs;* † *P < 0.05 Apoptotic cells in Oxytocin 10 nM group in Wistar rats versus Apoptotic cells in Oxytocin 10 nM group in SHRs;* # *P < 0.05 Necrotic cells in Oxytocin 100 nM group in Wistar rats vs Necrotic cells in Oxytocin 100 nM group in SHRs;* ## *P < 0.01 Necrotic cells in Control group in Wistar rats vs Necrotic cells in Control group in SHRs.*

4.3.4. Comparison of the effect of oxytocin and its pre-treatment with Ang II on the phosphorylation of ERK1/2, PI3K p110α, and AKT in aortic Wistar VSMCs and SHR VSMCs

Western blotting was utilized to study the effect of oxytocin, Ang II, and its pre-treatment with Ang II on the phosphorylation of ERK1/2, PI3K p110α, and Akt. The densitometric analysis of western blot images demonstrated that oxytocin and its pre-treatment with Ang II significantly decreased the phosphorylation of ERK1/2, PI3K p110 α, and Akt in aortic Wistar VSMCs and SHR VSMCs.

On the phosphorylation of ERK1/2

There was no significant difference between Wistar VSMCs and SHR VSMCs regarding oxytocin-mediated decrease in the levels of the phosphorylated forms of (active form) ERK1/2: oxytocin decreased the phosphorylation of ERK1/2 by 57.9% ± 14.2% in Wistar VSMCs and by 35.4% ± 4% in SHR VSMCs compared to respective controls (**Figure 4.30 b**).

Although, Ang II-induced phosphorylation of ERK1/2 was significantly higher in Wistar VSMCs ($P < 0.05$) than SHR VSMCs. In Wistar VSMCs, Ang II increased the phosphorylation of ERK1/2 by 223% ± 11% compared to control, whereas, in SHR VSMCs, Ang II increased the phosphorylation of ERK1/2 by 157% ± 21% compared to control (**Figure 4.30 a**).

The pre-treatment of oxytocin with Ang II caused more significant decrease in the phosphorylation of ERK1/2 in SHR VSMCs [93.3% ± 1.4% decrease ($P < 0.001$) than in Wistar VSMCs (76.6% ± 1.4% decrease) compared to their respective Ang II (alone) groups (**Figure 4.30 c**).

On the phosphorylation of PI3K p110α

There was a significant difference between oxytocin-mediated reduction in the phosphorylated form of (active form) of PI3K p110α between Wistar VSMCs and SHR VSMCs. Oxytocin showed a more substantial decrease of phospho-PI3K p110α in Wistar VSMCs ($P < 0.01$) than SHR VSMCs. In Wistar VSMCs, oxytocin reduced phospho-PI3K p110α by 64.8% ± 2.8% compared to control. On the other hand, in SHR VSMCs, oxytocin reduced phospho-PI3K p110α by 31% ± 5.1% compared to control (**Figure 4.30 e**).

Ang II-induced phosphorylation of PI3K p110α was significantly higher in Wistar VSMCs ($P < 0.01$) than SHR VSMCs. In Wistar VSMCs, Ang II increased phospho-PI3K p110α by 184% ± 17% compared to control, whereas, in SHR VSMCs, Ang II increased phospho-PI3K p110α by 79.8% ± 10.3% compared to control (**Figure 4.30 d**).

The oxytocin's pre-treatment with Ang II showed significantly more decrease in phospho-PI3K p110α levels in Wistar VSMCs ($P < 0.05$) than SHR VSMCs. The oxytocin's pre-treatment decreased the phosphorylation of PI3K p110α by 83.8% ± 8.6% in Wistar VSMCs and by 61.3% ± 4.8% in SHR VSMCs, respectively, compared to their respective Ang II (alone) groups (**Figure 4.30 f**).

On the phosphorylation of Akt

There was no significant difference in oxytocin-mediated decrease in the phosphorylation of Akt between Wistar VSMCs (46.2% ± 9.5% decrease) and SHR VSMCs (45.5% ± 8.6% decrease) compared to the respective control groups (**Figure 4.30 h**).

Similarly, Ang II-induced increase in the phosphorylation of Akt was not markedly different between Wistar VSMCs (44.4% ± 3.5%) rats and SHR VSMCs (70.2% ± 20.4% increase) compared to their respective control groups (**Figure 4.30 g**).

The pre-treatment of oxytocin with Ang II also didn't show any significant difference in inhibiting phospho-Akt between Wistar VSMCs (62.1% ± 10.9% decrease) and SHR VSMCs (60.4% ± 2.6% decrease) compared to the respective Ang II (alone) groups (**Figure 4.30 i**).

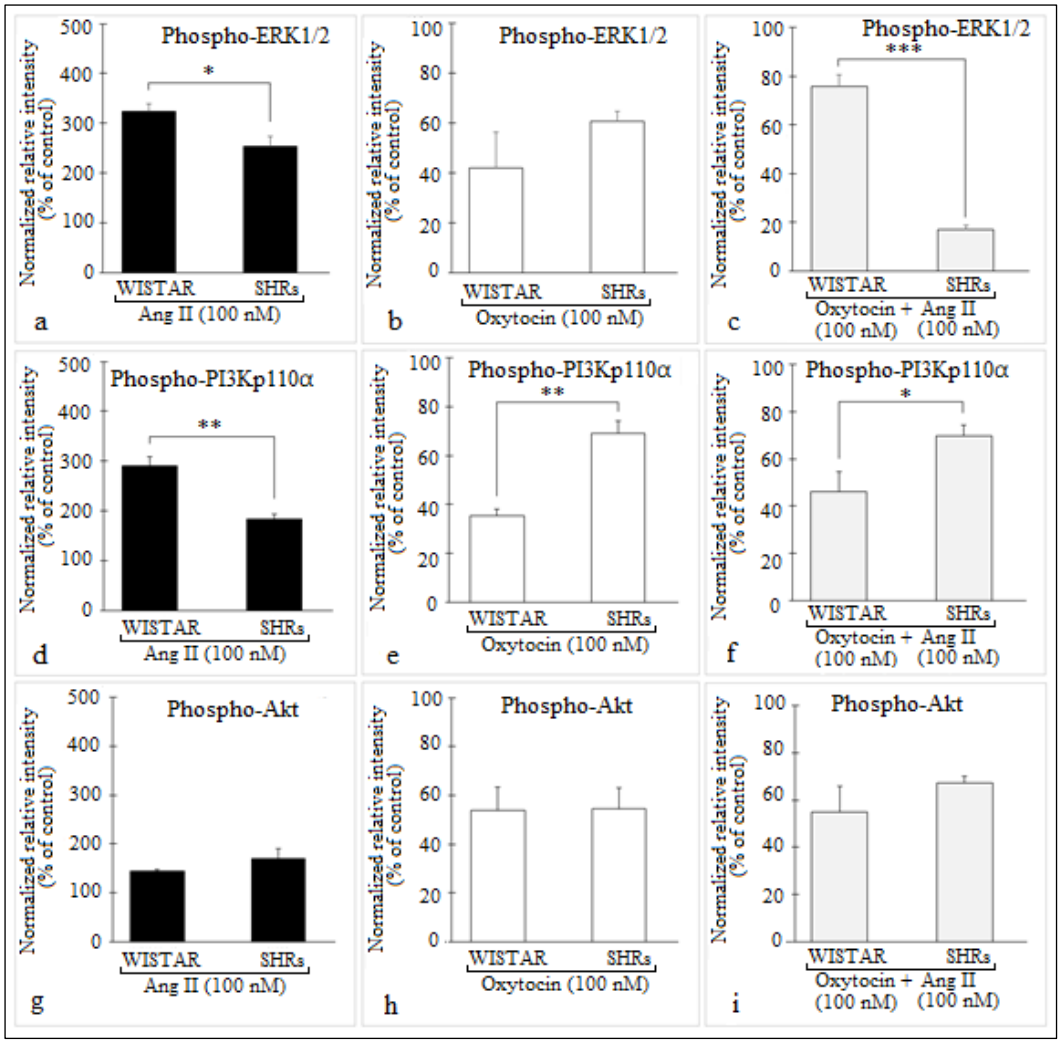

Figure 4.30. The comparison of the effects of oxytocin, Ang II, and pre-treatment of oxytocin with Ang II on the phosphorylation of ERK1/2 (figure a, b, and c), PI3K p110α (figure d, e, and f), and Akt (figure g, h, and i) in aortic Wistar and SHR VSMCs. *The data are shown as the normalized relative intensity of the bands and are represented as percent (%) of control, with control as 100%. The data are shown as the mean ± SEM of five (five Wistar rats and five SHRs each), three (three Wistar rats and three SHRs each), and four (four Wistar rats and four SHRs each) independent experiments conducted for ERK1/2, PI3K p110α, and Akt, respectively. * indicates $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Ang II indicates Angiotensin II; OXTA, oxytocin receptor antagonist; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol-3-kinase.*

4.3.5. Comparison of the effect of oxytocin and its pre-treatment with Ang II on vasocontraction pathway: ROCK-1 and ROCK-2 in aortic VSMCs isolated from Wistar rats and SHRs

Western blotting was employed to investigate the effect of oxytocin, Ang II, and its pre-treatment with Ang II on the activation of ROCK-1 and ROCK-2. The densitometric analysis of western blot images demonstrated that oxytocin significantly decreased the activation of ROCK-1 and ROCK-2 in both aortic Wistar VSMCs and SHR VSMCs.

On ROCK-1

There was no significant difference between oxytocin-mediated reduced activation of ROCK-1 between Wistar VSMCs and SHR VSMCs, respectively (**Figure 4.31**).

Oxytocin reduced the ROCK-1 activation by 60.4% ± 7.6% in Wistar VSMCs and by 58.9% ± 5.4% in SHR VSMCs compared to their respective control groups (**Figure 4.31 b**).

Similarly, Ang II-induced activation of ROCK-1 was not markedly different between Wistar VSMCs (147 % ± 50 % increase) and SHR VSMCs (102% ± 43% increase) compared to their respective control groups (**Figure 4.31 a**).

The pre-treatment of oxytocin with Ang II also didn't show any significant difference in reducing the activation of ROCK-1 between Wistar VSMCs (85.2% ± 10.6% decrease) and SHR VSMCs (79% ± 6.9% decrease) compared to the respective Ang II (alone) groups (**Figure 4.31 c**).

On ROCK-2

Similar to ROCK-1, the impact of oxytocin in reducing the activation of ROCK-2 was not markedly different between Wistar VSMCs and SHR VSMCs (**Figure 4.31**).

Oxytocin-mediated reduced activation of ROCK-2 in Wistar VSMCs (38.7% ± 13.1% decrease) was not statistically different from that in SHR VSMCs [(41.5% ± 12.8% decrease (**Figure 4.31 e**)]. Ang II-induced increased activation of ROCK-1 in Wistar VSMCs (83.2% ± 14.6% increase) was not prominently different from SHR VSMCs (81.2% ± 14.8% increase) compared to their respective control groups (**Figure 4.31 d**).

The pre-treatment of oxytocin with Ang II also didn't show any substantial difference in reducing the activation of ROCK-1 between Wistar VSMCs (65.8% ± 13.2% decrease) and SHR VSMCs (69.5% ± 13.2% decrease) compared to their Ang II (alone) groups respectively (**Figure 4.31 f**).

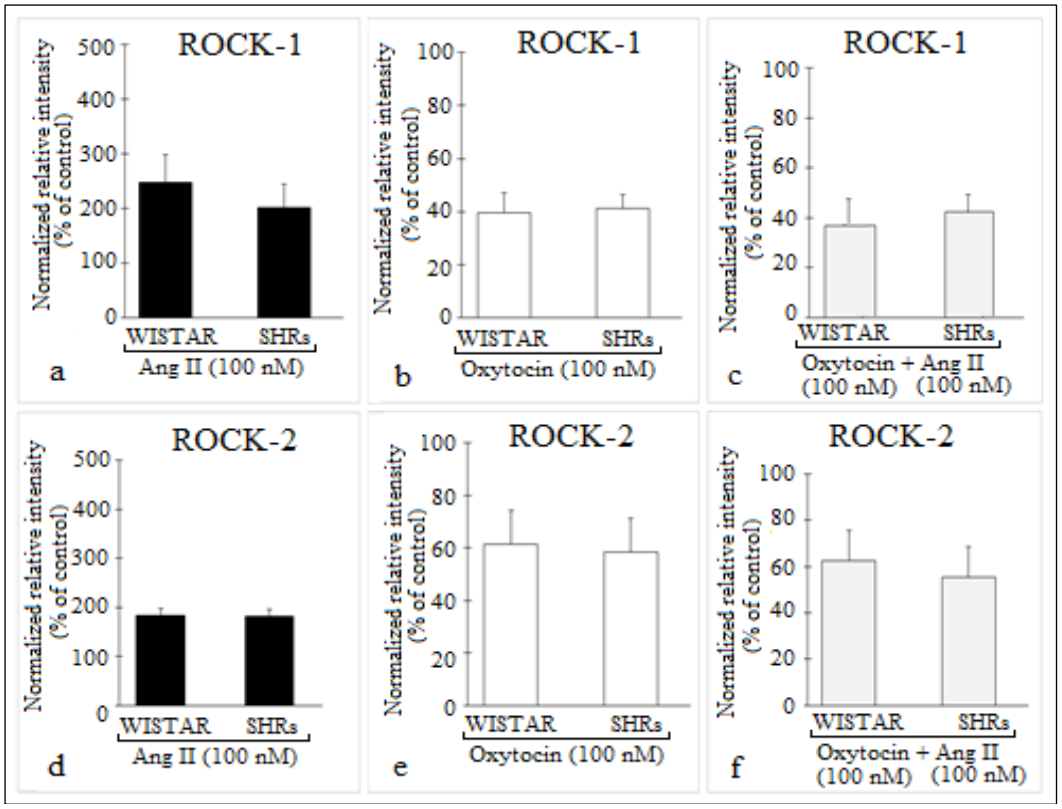

Figure 4.31. The comparison of the effects of oxytocin, Ang II, and the pretreatment of oxytocin with Ang II on the phosphorylation of Rho kinases – ROCK-1 (figure a, b, and c) and ROCK-2 (figure d, e, and f) in aortic Wistar and SHR VSMCs. *The data are shown as the normalized relative intensity of the bands and are represented as percent (%) of control, with control as 100%. The data are shown as the mean ± SEM of four (four Wistar rats and four SHRs each) and five (five Wistar rats and five SHRs each) independent experiments conducted for ROCK-1 and ROCK-2, respectively. Ang II indicates Angiotensin II; OXTA, oxytocin receptor antagonist; ROCK, rho-associated coiled-coil containing protein kinase.*

4.3.6. Comparison of the effect of oxytocin and its pre-treatment with Ang II on IL-6 in aortic Wistar VSMCs and SHR VSMCs

The ELISA based IL-6 assay involved the measurement of the concentration of IL-6 released in the culture media. The findings of IL-6 assay established that oxytocin significantly increased IL-6 secretion from aortic VSMCs, but its pre-treatment with Ang II significantly decreased IL-6 in both Wistar VSMCs and SHR VSMCs.

There was a significant difference in IL-6 secretion levels in the control group between Wistar VSMCs and SHR VSMCs. The control group in SHR VSMCs showed more IL-6 [848 ± 9 pg/mL ($P < 0.05$)] than Wistar VSMCs [614 ± 104 pg/mL (**Figures 4.32** and **4.33**)].

The assay employed three different concentrations of oxytocin: 10 nM, 100 nM, 1000 nM. In response to different concentrations of oxytocin, aortic SHRs VSMCs showed significantly more IL-6 secretion [985 ± 25 pg/mL ($P < 0.05$) at 10 nM (**Figure 4.32**); 1,289 ± 8 pg/mL ($P < 0.001$) at 100 nM (**Figures 4.32** and **4.33**); and 1,468 ± 19 pg/mL ($P < 0.001$) at 1,000 nM (**Figure 4.32**)] than Wistar VSMCs (695 ± 101 pg/mL at 10 nM; 1033 ± 24 pg/mL at 100 nM; and 959 ± 52 pg/mL at 1,000 nM) compared to their respective controls.

There was no significant difference between Wistar VSMCs and SHR VSMCs with regards to Ang II-induced secretion of IL-6 (1,481 ± 91 pg/mL in Wistar VSMCs and 1,620 ± 66 pg/mL in SHR VSMCs) compared to their respective control groups (**Figures 4.32** and **4.33**).

Although oxytocin on its own significantly increased IL-6 in both Wistar VSMCs and SHR VSMCs, interestingly, oxytocin's pre-treatment (100 nM) followed by Ang II (100 nM) significantly decreased IL-6 secretion in both Wistar VSMCs (735 ± 77 pg/mL) and SHR VSMCs (891 ± 59 pg/mL) compared to their respective Ang II 100 nM (alone) groups (**Figures 4.32** and **4.33**).

However, there was no significant difference between the Wistar VSMCs and SHR VSMCs concerning the effect of oxytocin's pre-treatment on Ang II-induced IL-6 secretion (**Figures 4.32** and **4.33**). The pattern and magnitude of the impact of oxytocin pre-treatment on Ang II-mediated IL-6 secretions were similar in both Wistar VSMCs and SHR VSMCs.

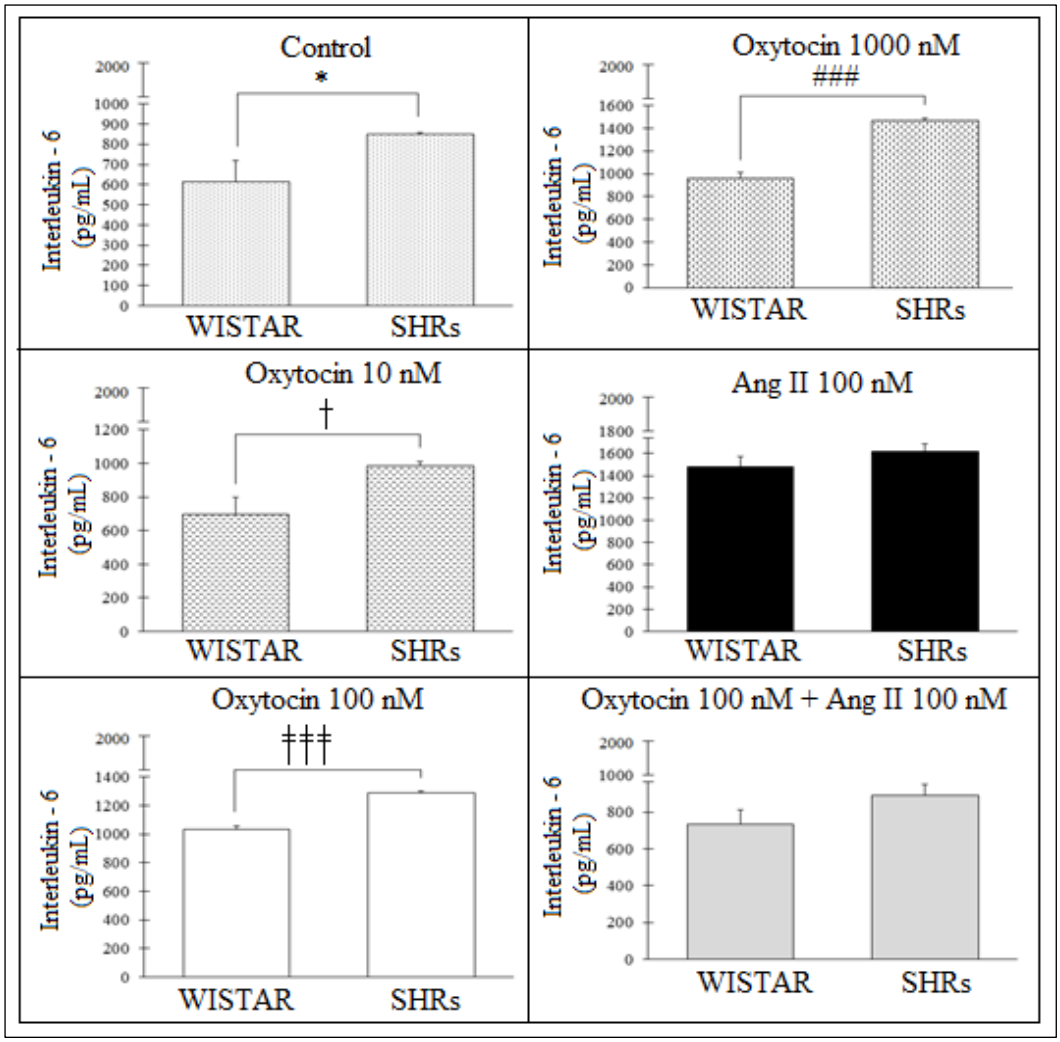

Figure 4.32. The comparison of the effect of oxytocin (10 nM, 100 nM, and 1,000nM), Ang II (100 nM), pre-treatment of oxytocin with Ang II on IL-6 secretion in aortic Wistar VSMCs and SHRs VSMCs. *The concentration of IL-6 secretion by the aortic VSMCs in the culture media was measured in picogram per mL (pg/mL). The data are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three Wistar rats and three SHRs, respectively. In each experiment each treatment group had duplicates.* ***** *indicates* $P < 0.05$ *Control Wistar rats versus Control SHRs;* †$P < 0.05$ *Oxytocin 10 nM Wistar rats versus Oxytocin 10 nM SHRs;* ‡‡‡ $P < 0.001$ *Oxytocin 100 nM Wistar rats versus Oxytocin 100 nM SHRs;* $^{###}$ $P < 0.001$ *Oxytocin 1000 nM Wistar rats versus Oxytocin 1000 nM SHRs. Ang II indicates Angiotensin II.*

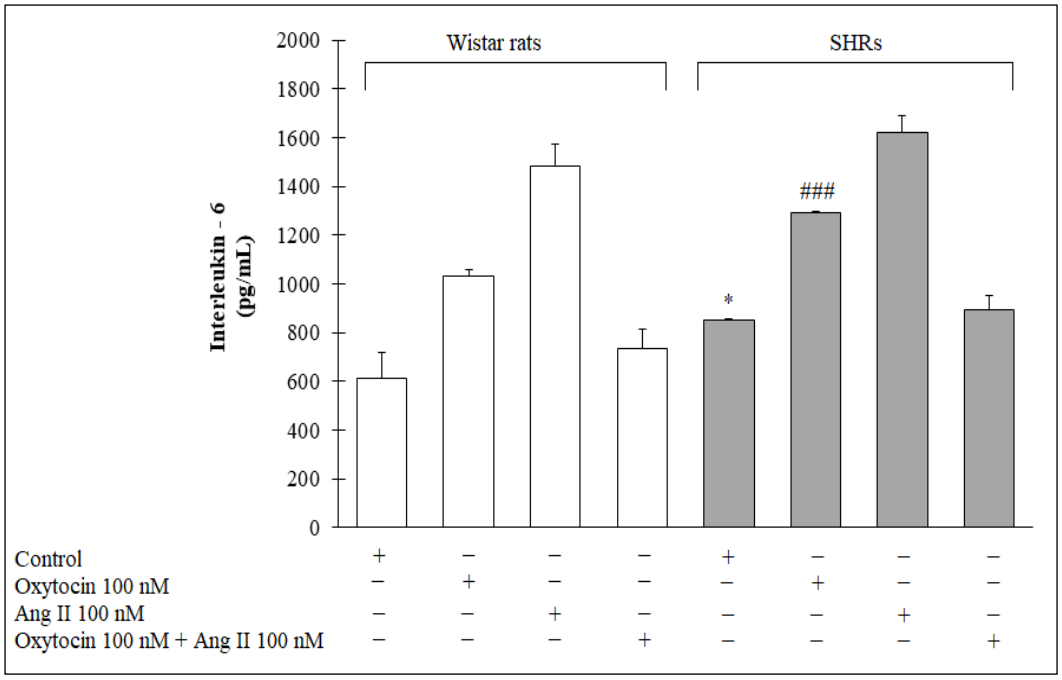

Figure 4.33. The comparison of the effect of oxytocin (100 nM), Ang II (100 nM), pre-treatment of oxytocin (100 nM) with Ang II (100 nM) on IL-6 secretion in aortic Wistar VSMCs and SHRs VSMCs. *The concentration of IL-6 secreted by the aortic VSMCs in the culture media was measured in picogram per mL (pg/mL). The data are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three Wistar rats and three SHRs, respectively. In each experiment each treatment group had duplicates.* ***** *indicates* $P < 0.05$ *Control group Wistar rats versus Control group SHRs;* $^{###}$ $P < 0.001$ *Oxytocin 100 nM in Wistar rats versus Oxytocin 100 nM in SHRs. Ang II indicates Angiotensin II.*

4.3.7. Comparison of the effect of oxytocin and its pre-treatment with Ang II on ROS production in aortic Wistar VSMCs and SHR VSMCs

The ROS assay findings demonstrated that oxytocin significantly increased the ROS production in aortic VSMCs isolated from both Wistar rats and SHRs.

In addition, the pre-treatment of oxytocin followed by Ang II significantly increased ROS production in both Wistar VSMCs and SHR VSMCs. However, there was a significant difference in the impact of oxytocin and its pre-treatment with Ang II on ROS production between Wistar VSMCs and SHR VSMCs.

The control group Wistar VSMCs showed more ROS levels [7,542 ± 128 ($P < 0.001$)] compared to the control group SHR VSMCs [5,411± 259 (**Figure 4.44**)].

The treatment with oxytocin (100 nM) resulted in a significantly more ROS production in Wistar VSMCs [8,584 ± 114 ($P < 0.001$)] than SHR VSMCs (6,657 ± 202) compared to their respective controls (**Figure 4.44**).

Ang II (100 nM)-induced ROS production was significantly higher in Wistar VSMCs [8,336 ± 204 $P < 0.001$)] than SHR VSMCs [6,286 ± 208 (**Figure 4.44**)].

The pre-treatment of oxytocin (100 nM) followed by Ang II (100 nM) also showed significantly higher ROS production [9,346 ± 143 $P < 0.001$)] in Wistar VSMCs than SHR VSMCs (7,481 ± 162) compared to their respective Ang II (alone) and Oxytocin groups (alone), respectively (**Figure 4.44**).

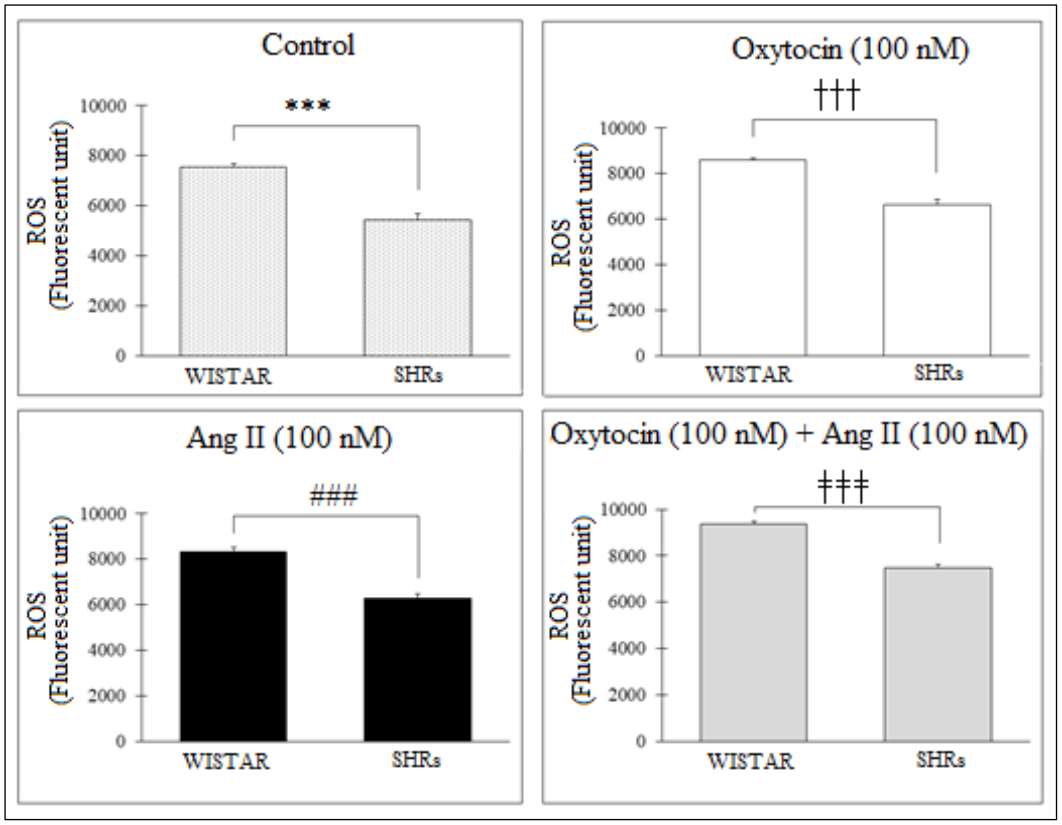

Figure 4.44. The comparison of the effect of oxytocin, Ang II, pre-treatment of oxytocin with Ang II on ROS production between aortic Wistar and SHR VSMCs. *VSMCs were seeded at 1×10^4 cells/well in 96-well plates. The ROS levels were measured as the function of the fluorescent unit. The data are represented as the mean ± SEM from three independent experiments performed using aortic VSMCs from three Wistar rats and three SHRs, respectively. In each experiment each treatment group had 12 replicates. *** indicates $P < 0.001$ Control group in Wistar rats versus Control group in SHRs; †††$P < 0.001$ Oxytocin 100 nM in Wistar rats versus Oxytocin 100 nM in SHRs; ### $P < 0.001$ Ang II 100 nM in Wistar rats versus Ang II 100 nM in SHRs; ‡‡‡ $P < 0.001$ Oxytocin 100 nM + Ang II 100 nM in Wistar rats versus Oxytocin 100 nM + Ang II 100 nM in SHRs. Ang II indicates Angiotensin II.*

Chapter 5: Discussion

CVD is the leading cause of hospitalization in the United States and accounts for mortality more than other any other disease, including cancer and communicable diseases.[263] Each year about 17 million deaths occur due to CVD, which is two times higher than deaths caused by cancer, and in all probability, this number is going to increase to about 23.3 million by 2030.[264]

Despite advancement in diagnosis and improvement in efforts aimed at prevention, control, and treatment, CVD remains a primary cause of premature death and disability across the globe.[265] Consequentially, CVD healthcare management has imposed an enormous financial burden on patients.[266]

The current standard CVD management includes therapies aimed at controlling patients' lipid-profiles (statins), blood glucose levels (metformin), and prescription of anti-hypertensive (ACE inhibitors) and anti-platelet medications (aspirin).[267] However, lesser favorable patient outcomes, as well as the rapidly rising rate of mortality due to CVD, paint a different picture. It reflects a growing need to expand CVD research so that new therapeutic options can be devised to effectively treat CVD. The present study was a step towards exploring the potential of oxytocin in CVS.

Over decade oxytocin, which is often referred to as a female reproductive hormone,[1,9,268,269] has garnered the attention of the scientific community as a hormone with cardioprotective effects.[23,24] Much of the research so far has focused on oxytocin-mediated

protective effects in cardiomyocytes, vascular endothelial cells, and cardiogenesis.[247,30,32,40] Besides identifying the presence of the oxytocin receptors in VSMCs,[25] not much research has been done concerning the potential effect of oxytocin on VSMCs.

VSMCs play a crucial role in cardiovascular physiology and are often at the center of the events in the pathophysiology of CVD including hypertension.[270] They undergo increased cell proliferation in response to hypertension-induced stress on the vasculature, leading to hypertrophy and an aberrant increase in vascular contraction.[271,80] These changes are mediated by complex signaling pathways mainly the ERK1/2 pathway,[99] the PI3K/Akt pathway,[105] and ROCK pathway.[144] In addition, increased oxidative stress and prolonged inflammation add to and exacerbate injury to VSMCs, and manifest the long-term pathological changes in vasculature.[272,273]

Interestingly, the interaction of oxytocin with other vital peptides playing crucial roles in CVD such as Ang II has not been well-researched. Ang II is the primary effector hormone and the central component of the RAAS. It causes vasoconstriction and maintains blood pressure in normal physiological conditions.[50]

Ang II is also involved in CVD such as atherosclerosis, congestive heart failure, diabetes, hypertension, inflammation, and myocardial infarction.[51] It mainly binds to AT1R in the CVS and executes its actions via activation of the ERK1/2 pathways (increasing phospho-ERK1/2),[274,275] PI3K/Akt pathway (phosphorylating PI3K p110α and increasing phospho-Akt)[276,277] and ROCK pathway (activation of ROCK-1 and ROCK-2).[278,279] These signaling pathways and kinases are involved in Ang II-induced hypercontraction of VSMCs, hypertrophy, hyperplasia, endothelial dysfunction, leading to

vascular diseases and their progression.[53,54] In addition, in cardiovascular pathophysiological conditions Ang II precipitates inflammation and oxidative stress.[280,59]

We hypothesized that oxytocin provides vascular protection in VSMCs via modulation of these pathways, inflammation, and reduction of oxidative stress, and antagonization of the Ang II effects on these pathways, inflammation and oxidative stress. We investigated oxytocin's effect on cell proliferation, inflammation, oxidative stress and on vasocontraction pathway in primary aortic VSMCs, isolated from the adult normotensive Wistar rats and SHRs.

5.1. Oxytocin reduced the proliferation in aortic VSMCs

The findings of the present study demonstrated that oxytocin significantly decreased cell proliferation of normotensive (Wistar) and hypertensive (SHR) VSMCs. This is in contrast with the proliferative effect of oxytocin on vascular endothelial cells derived from: immortalized human dermal microvasculature, breast-tumors, human umbilical vein; and cardiomyocytes.[40,41]

It is noteworthy that the kind of effect oxytocin shows on cell proliferation depends on the type of cells involved. While previous studies have shown oxytocin exerting a proliferative influence on, for instance, trophoblast and astrocytes, others have shown oxytocin mediating anti-proliferative effects on cell lines obtained from breast cancer, ovarian cancer, and prostate cancer.[281–283]

Even within the cell lines derived from a similar source but from different stages of differentiation, oxytocin has shown a differential effect on proliferation. For example, in osteosarcoma cell lines (SaOS-2, TE-85, and UMR-106) obtained from different stages of differentiation, oxytocin showed dual action on proliferation. Oxytocin increased cell proliferation in SaoS-2 cells and decreased cell proliferation in both TE-85 cells and UMR-106 cells.[284]

Although only one type of oxytocin receptor has been found across species,[3] these dual effects of oxytocin on proliferation suggest the probability of another subtype of oxytocin receptor.

In addition to showing anti-proliferative effects of oxytocin on aortic VSMCs, the outcomes of the present study have revealed that oxytocin significantly reduced Ang II-induced cell proliferation of aortic VSMCs isolated from

normotensive Wistar rats. Importantly, this effect was consistent in hypertensive SHRs VSMCs. Further, the oxytocin receptor antagonist significantly attenuated both these effects, suggesting that oxytocin mediated these effects via its receptors.

It is essential to understand the impact of oxytocin-induced inhibition of Ang II-mediated proliferation of SHR VSMCs in the broader context of CVD pathophysiology.

In hypertension induced by hyperactivation of Ang II signaling, VSMCs undergo extensive remodeling including hypertrophy and fibrosis. Moreover, cardiomyocytes in the left ventricle of the heart undergo hyper-proliferation leading to cardiac hypertrophy and ultimately, over time, compromise the function of heart and blood vessels.[285] These events lay the foundation for a cascade of numerous other intra-vascular and intra-cardiac pathophysiological events worsening CVD.[286]

Evidence that oxytocin's anti-proliferative action on VSMCs and oxytocin-induced decrease in Ang II-mediated proliferation of SHR VSMCs generated from this study underline the potential oxytocin might have in vascular protection in CVD such as hypertension, and this merits further investigation.

5.2. Oxytocin deactivated the proliferation signal transduction pathways in aortic VSMCs

For greater understanding about how oxytocin reduces cell proliferation in aortic VSMCs, this study explored the two key downstream cell signaling pathways involved in the proliferation of VSMCs: the ***ERK1/2 MAPK pathway*** and the ***PI3K/Akt pathway***.

This study results revealed that oxytocin significantly reduced the phosphorylation of ERK1/2 in normotensive and hypertensive VSMCs. This is in contrast with reports from previous studies, which reported that oxytocin treatment increased phosphorylation of ERK1/2 in cardiomyocytes and vascular endothelial cells.[40,102]

The differential effects of oxytocin on the phosphorylation of ERK1/2 have been proposed to be based on the type of cells involved, location of oxytocin receptors, activation of the different G-proteins, and interaction with a phosphatase. For example, oxytocin demonstrated dual effects on phosphorylation of ERK1/2 in human embryonic kidney cells. These effects were based on the site of oxytocin receptors. Oxytocin decreased the phosphorylation of ERK1/2 when oxytocin receptors were present outside the caveolin-1-enriched microdomains and resulted in reduced cell proliferation. And, oxytocin increased the phosphorylation of ERK1/2 when oxytocin receptors were present inside the caveolin-1-enriched microdomains and henceforth, increased cell proliferation.[103]

It is worth stating that caveolin-1 is vital in the trafficking of $G\alpha_s$ (stimulatory) protein, inhibition of $G\alpha_s$/adenylyl cyclase pathway,[104] and is also thought to be involved in the regulation of cell proliferation and cell survival.[287]

In another study, oxytocin caused the dephosphorylation of eukaryotic elongation factor 2 (eEF2) and contributed to an increase in protein translation and synthesis in myometrial cells via PKC.[288] The eEF2 kinase is responsible for the phosphorylation of eEF2. The phosphorylated form of eEF2 is inactive and causes downregulation of protein translation, whereas, the dephosphorylated form is the active form and leads to an increase in protein translation and synthesis. The eEF2 kinase is regulated by a phosphatase known as protein phosphatase 2 A (PP2A).[289]

In addition to oxytocin inducing dephosphorylation of eEF-2 through PKC,[288] it could very well be that oxytocin might increase the phosphatase (PP2A) leading to reduced activity of eEF2 kinase and thus inducing dephosphorylation of eEF2. Furthermore, oxytocin reduced Ang II-induced phosphorylation of ERK1/2, and these effects were significantly abolished by pre-treatment with an oxytocin receptor antagonist. Interestingly, these effects of oxytocin were consistent in aortic VSMCs isolated from hypertensive rats.

The significance of the study results demonstrating oxytocin-induced inhibition of phosphorylation of ERK1/2 and oxytocin-mediated inhibition of Ang II-induced ERK 1/2 phosphorylation in SHR VSMCs can be understood by assessing the implication of the ERK1/2 signaling pathway in CVD pathophysiology.[290]

An upregulation of agonist (Ang II)-stimulated ERK1/2 signaling for prolonged duration has been shown to induce pathological hypertrophy in VSMCs and decompensated cardiac hypertrophy in the heart.[291] Thus, the ERK 1/2 signaling pathway plays a crucial role in the precipitation of CVD, for example, hypertension, myocardial infarction, and stroke.[99,]

The present study provided evidence, that by decreasing the phosphorylation of ERK1/2, oxytocin deactivated Ang II-stimulated ERK1/2 signaling in SHR VSMCs, and thus supports a protective role of oxytocin in pathophysiological conditions, for instance, hypertension. However, in vivo studies are required to further shed more light on oxytocin's protective effect on hypertension.

The PI3K/Akt signaling pathway has been shown to alter vascular function by increasing the myogenic tone of resistant arteries.[107] It also results in an aberrant increase in the activity of L-VGCC leading to increased intracellular calcium, increased cell volume in cardiomyocytes, and hyper-proliferation of VSMCs.[120,110] Thus, the PI3K/Akt pathway leads to various cardiovascular pathophysiological conditions including hypertension, pathological cardiac hypertrophy, and cardiac arrhythmia .[119] Ang II has been known to participate in and worsen these pathophysiological events via hyper-activation of the PI3K/Akt signaling. [276,53]

The findings of this study showed that oxytocin remarkably reduced the phosphorylation of PI3K p110α and Akt in aortic VSMCs isolated from normotensive and hypertensive rats. These findings are consistent with a previous

study, wherein oxytocin reduced the phosphorylation of PI3K p110α and Akt, albeit in gut cells.[292]

Similar to the effect of oxytocin on Ang II-induced induced phosphorylation of ERK1/2, oxytocin prominently diminished Ang II-induced phosphorylation of PI3K p110α and Akt, whereas, the oxytocin receptor antagonist prevented these effects of oxytocin. These findings were also consistent in aortic SHR VSMCs, which further suggests that oxytocin induced-deactivation of PI3K/Akt signaling pathway in SHR VSMCs might provide vascular protective effects in hypertension in vivo.

There is a possibility that in aortic VSMCs, oxytocin via its receptors, which belong to GPCRs superfamily,[3] might be recruiting $G\alpha_i$ (inhibitory) protein, and thus inhibiting downstream cell signaling by reducing the levels of phospho-ERK1/2, phospho-PI3K p110α, and phospho-Akt, leading to the deactivation of the ERK1/2 pathway and the PI3K/Akt pathway.

Alternatively, it could very well be that oxytocin might regulate the phosphatase and thereby reduce the phosphorylation of ERK/12, PI3K p110α, and Akt.

5.3. Oxytocin deactivated the ROCK pathway in aortic VSMCs

The present study explored the effect of oxytocin on the ROCK pathway. The agonist-mediated phosphorylation of Rho kinases leads to the activation of both ROCK-1 and ROCK-2, along with concurrent calcium mobilization, causing the organization and remodeling of the actin cytoskeleton, extracellular matrix, and contraction of VSMCs.[146] The ROCK signaling pathway has been implicated in the pathogenesis of various CVD such as hypertension, pulmonary hypertension, angina pectoris, stroke, and heart failure. [140–144] It is also established that Ang II activates ROCK signaling in these pathophysiological conditions.[135,53]

Together with the reduction in cell proliferation and deactivation of the proliferation signal transduction pathways by oxytocin, the present research demonstrated for the first time that oxytocin significantly reduced Rho kinase-mediated vasocontraction in aortic VSMCs. The study results highlight that oxytocin reduced the activation of both ROCK-1 and ROCK-2 in aortic VSMCs isolated not only from normotensive rats but also from hypertensive rats. These findings suggest that oxytocin could provide vascular protective effects in pathophysiological conditions associated with hyper-signaling of ROCK pathways such as hypertension and stroke.

However, to state definitively about oxytocin's anti-vasocontraction effect on VSMCs, direct measurement of oxytocin's impact on vasocontraction would be needed. The study was limited in this regard as this study did not directly assess oxytocin's effect on vasocontraction. Instead, it investigated the impact of oxytocin on the vasocontraction pathway (ROCK pathway). Future studies could be carried

out by employing an ex vivo model such as the organ bath to directly measure vasocontraction.

The study outcomes further revealed that oxytocin reduced Ang II-mediated activation of both ROCK-1 and ROCK-2 in normotensive as well as in hypertensive VSMCs, and these effects were blocked by an oxytocin receptor antagonist, indicating that oxytocin causes these effects via its receptors. Based on the study results, the fact that oxytocin antagonized Ang II-stimulated ROCK pathway in aortic VSMCs suggests the potential cardioprotective effects oxytocin might have in conditions where Ang II-mediated hyper-signaling of ROCK causes vasoconstriction of blood vessels and disrupts the hemodynamics, culminating in the development and worsening of CVD such as hypertension, angina pectoris, and stroke.

This study was the first attempt to investigate the impact of oxytocin and its pre-treatment with Ang II on the ROCK pathway. However, future in vivo studies would be needed to further explore oxytocin's effect on the contraction of blood vessels in normal and pathophysiological conditions, for example, hypertension.

All three pathways: the ERK1/2 pathway, the PI3K/Akt pathway, and the ROCK1/ROCK2 pathway are crucial in precipitating deleterious events such as hypertrophy and hyper-contractility in VSMCs, which further lead to cardiovascular complications manifested in CVD such as hypertension and arrhythmia.[99,119,144]

Our results indicate that oxytocin may prevent these changes in the dynamics of the vasculature by reducing the phosphorylation of ERK1/2, PI3K, and

Akt and by decreasing the activation of ROCK-1 and ROCK-2 in normotensive as well as hypertensive conditions (**Figure 5**).

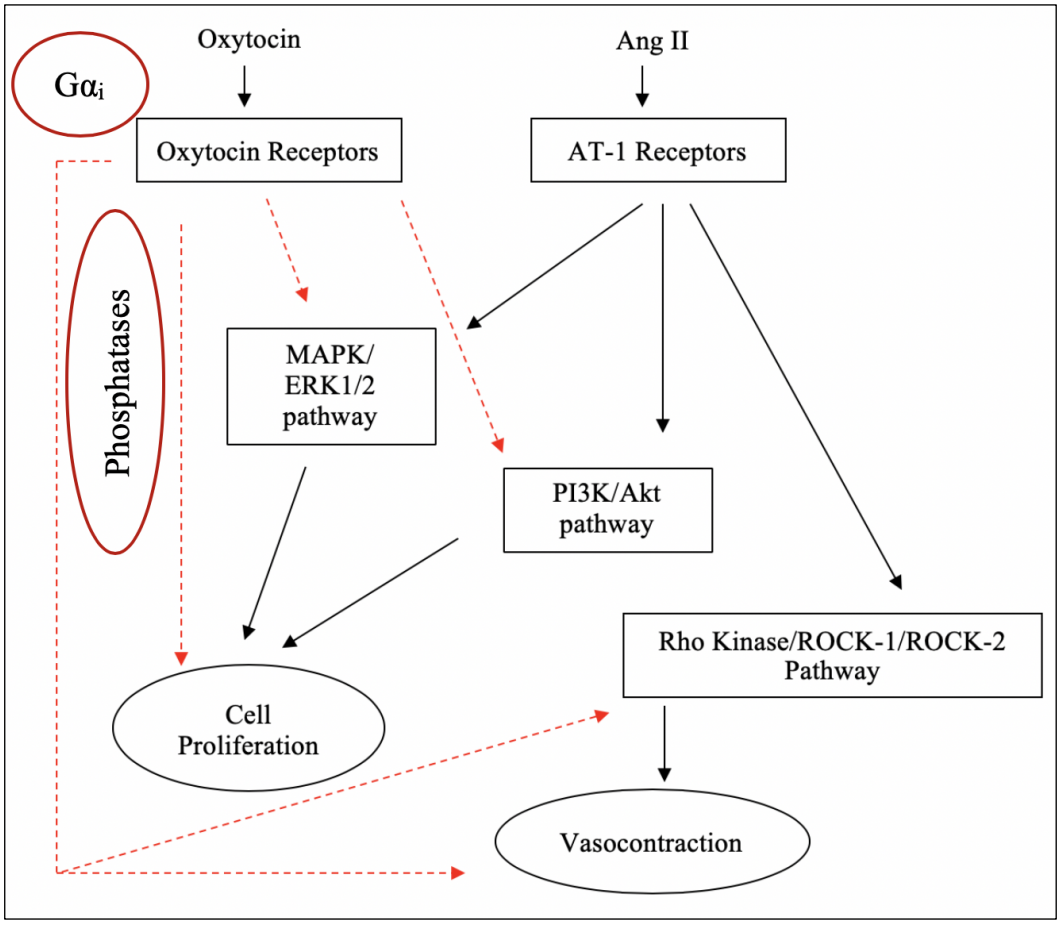

Figure 5. Inhibition of the ERK1/2 pathway, the PI3K/Akt pathway, and the Rho-kinase pathway by oxytocin in aortic VSMCs. *Oxytocin mediates anti-proliferative effects by inhibiting phosphorylation of ERK1/2, PI3K, and Akt. Oxytocin deactivates Rho-kinase pathway involved in vasocontraction of VSMCs by inhibiting the phosphorylation of ROCK-1 and ROCK-2. It may be possible that either via the recruitment of $G\alpha_i$ proteins or by stimulating phosphatases oxytocin decreases phosphorylation of ERK1/2, PI3K, Akt, ROCK-1, and ROCK-2. ERK indicates extracellular signal-regulated kinase; PI3K, phosphoinositide-3-kinase; ROCK, rho-associated coiled-coil containing protein kinase; Ang II, angiotensin II; AT-1, angiotensin type-1; $G\alpha_i$, G alpha inhibitory protein. Dotted red arrows indicate inhibition; black arrows, activation.*

5.4. Oxytocin increased IL-6 in aortic VSMCs

One of the objectives of the current study was to investigate the effects of oxytocin on inflammation responses using aortic VSMCs. Hence, IL-6, a biomarker of inflammation, was measured.

In contrast to previous studies carried out on vascular endothelial cells,[34] the outcomes of the present study, conducted on aortic VSMCs, revealed that oxytocin significantly increased IL-6, but remarkably reduced Ang II-stimulated IL-6 levels.

It is essential to understand the role of IL-6 while interpreting these results. IL-6 has been known for its pleiotropic effects.[172] It is involved in various physiological events including inflammation (production of CRP) and immunological responses (activation and differentiation of T-cells and B-cells).[174,175,178,180,181]

Several studies have shown that an increase in IL-6 levels by any treatment(s) or peptide(s) under investigation can be detrimental, especially in conditions, for example rheumatoid arthritis and heart disease, wherein IL-6 mediated immunological and pathophysiological events contribute in worsening of the symptoms.[293,294]

However, the effects of IL-6 can be both harmful and protective.[192] It is the duration of the pathogenic stimuli or pathophysiological conditions that determines which one of the two effects IL-6 will manifest. For example, IL-6 elicits cardioprotective effects on cardiomyocytes when exposed to acute pathophysiological condition(s).[192]

Conversely, IL-6 signaling can prove detrimental if the exposure to pathophysiological condition(s) is chronic. In such a scenario, IL-6 may result in pathological changes, for instance, reduced cardiac contractility, pathological cardiac hypertrophy, and ultimately leading to decreased cardiac function.[197,198]

In this context, based on this study findings, oxytocin-induced IL-6 secretion in VSMCs could be vascular protective, as oxytocin-induced IL-6 secretion might boost the immunological defense required to counter the pathogenic stimulus for a shorter period. The fact that oxytocin's effect on IL-6 was consistent in VSMCs isolated from hypertensive rats suggests oxytocin could similarly bolster immune defense against an acute exposure to a pathogen in diseased condition. However, further in vivo studies are required to explore the beneficial effect of oxytocin-induced IL-6 secretion against an acute exposure to pathogenic stimuli.

Another aspect of the present study was to determine the impact of oxytocin on Ang II-stimulated IL-6 secretion. The study results demonstrated that oxytocin reduced Ang II-stimulated IL-6 secretion not only in aortic VSMCs from normotensive rats but also in aortic VSMCs from hypertensive rats, indicating that oxytocin could show protective effects against Ang II-induced inflammation (increase in IL-6), which more likely happens in a chronic basis under cardiovascular disease conditions such as atherosclerosis and rheumatoid arthritis.

Based on the study findings that the singular treatment of oxytocin increased IL-6 secretion, it can be suggested that oxytocin might provide beneficial effects in conditions where a short-term inflammatory response is required to arrest the host

damage, for example, in cardiomyocytes where short-term IL-6 signaling provided cardioprotective effects.[192] Furthermore, oxytocin's antagonistic action against Ang II-induced IL-6 secretion could provide protection against chronic inflammation such as in coronary heart disease and atherosclerosis. [293,294]

5.5. Oxytocin increased ROS in aortic VSMCs

The current study was carried out to determine the effect of oxytocin on oxidative stress. Oxidative stress is often at the center of many pathophysiological conditions in the CVS.[295] It is caused by an imbalance between oxidants and ant-oxidants.[296] The oxidants are ROS such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-). On the other hand, antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRx).[297]

In cardiovascular cells such as VSMCs, endothelial cells, and cardiomyocytes, ROS is primarily produced by NADPH oxidase.[217] Others include mitochondrial transport chain and uncoupling of eNOS.[225,227] The vasoconstrictor peptides such as Ang II and ET -1 increase oxidative stress via increased production of ROS, contributing to the pathogenesis of CVD including hypertension.[219,221]

The results of the ROS measurement in the present study showed that oxytocin increased ROS production in aortic VSMCs. These results are in contrast with the effect of oxytocin on ROS production in vascular endothelial cells as reported in previous studies.[34] Also, oxytocin's pre-treatment with Ang II further increased ROS production, suggesting an additive effect. These effects of oxytocin were consistent in SHR VSMCs. The basal levels of ROS were higher in Wistar VSMCs, and consequently, oxytocin-induced ROS production was higher in Wistar VSMCs than SHR VSMCs.

These results suggest that oxytocin increases oxidative stress and further potentiate Ang II-induced oxidative stress in cultured aortic VSMCs. However, it

is essential to mention that the current study employed the in vitro experimental models, the effect of oxytocin was measured on isolated aortic VSMCs, of which the findings showed oxytocin increased ROS production. It cannot be assumed that oxytocin would show similar effects in vivo, where VSMCs are not separate and co-exist with endothelial cells.

It is worth noting endothelial cells, VSMCs, and their interaction plays a crucial role in maintaining vascular homeostasis. However, events such as increased ROS production causing oxidative stress and reduced availability of NO can lead to endothelial dysfunction. This can adversely impact the functions of VSMCs. VSMCs in such conditions are prone to excessive proliferation and hyper-contraction owing to lack of vasorelaxants (NO) and can lead to the development of CVD, for example, atherosclerosis.[298]

As stated earlier, oxytocin has been shown to induce protective effects in vascular endothelial cells via reduction of ROS production. It might be that in vivo, where both endothelial cells and VSMCs co-exist, oxytocin-mediated opposite effects on ROS production in VSMCs and endothelial cells might negate each other. This might not disrupt the overall redox equilibrium and may not adversely impact oxidative stress in blood vessels. This could be useful in conditions associated with increased oxidative stress such as hypertension, atherosclerosis, and rheumatoid arthritis.

However, the present study did not investigate the interaction between endothelial cells and VSMCs with regards to oxytocin's effect on ROS production, and this could be a subject of future investigation where oxytocin's effect on ROS

production in VSMCs and endothelial cells can be determined simultaneously by employing co-cultures of VSMCs and endothelial cells and in vivo studies.

5.6. Summary of the dissertation research

In the last decade and a half, the status of oxytocin as a contractor of the pregnant uterus, and an initiator of milk-ejection reflex have transcended to a hormone eliciting protective effects in the CVS, in general, and on cardiovascular cells including cardiomyocytes and vascular endothelial cells, in particular. However, oxytocin's impact on VSMCs, which form an integral layer of blood vessels and play a crucial role in physiological and pathophysiological conditions associated with CVS, have not been well-established. This study was carried out to investigate oxytocin's effect on proliferation, oxidative stress, inflammation, and on vasocontraction pathway in aortic VSMCs isolated from both normotensive and hypertensive rats.

In addition, this study also examined the interaction of oxytocin with Ang II, a vital hormone of the RAAS in the CVS. Ang II-induced proliferation, oxidative stress, inflammation, and vasocontraction of VSMCs have been associated with the development and worsening of various vascular diseases, including hypertension and atherosclerosis. This study investigated the effect of oxytocin's pre-treatment on Ang II-induced proliferation, oxidative stress, inflammation, and Ang II-stimulated vasocontraction pathway in VSMCs in aortic VSMCs isolated from both normotensive and hypertensive rats.

The present study employed primary aortic VSMCs isolated from normotensive Wistar rats and hypertensive SHRs. For investigating the effect of oxytocin, and its interaction with Ang II, on the proliferation of VSMCs, this study utilized various methods: Hemocytometer method for cell counting; MTT assay for

cell viability assessment; ^{3}H-Thymidine incorporation assay for cell growth measurement; and flow cytometry to determine apoptosis and necrosis.

To gain a better understanding of the impact of oxytocin on VSMCs proliferation, the two key proliferation signal transduction pathways were investigated: the ERK1/2 pathway and the PI3K/Akt pathway. Western blot technique was utilized to study the effect of oxytocin on these two pathways. For studying the impact of oxytocin, and its interaction with Ang II, on the vasocontraction pathway in VSMCs, the ROCK pathway was investigated. The western blot technique was utilized to study oxytocin's effect on the ROCK pathway.

To investigate the effect of oxytocin, and its interaction with Ang II, on inflammation and oxidative stress, the ELISA-based IL-6 assay, and the fluorescent spectroscopy-based ROS assay were carried out, respectively.

The outcomes of proliferation studies indicated that oxytocin reduces VSMCs proliferation by inhibiting the phosphorylation of ERK1/2, PI3K p110α, and Akt. Besides, oxytocin also reduced Ang II-induced proliferation by reducing Ang II-mediated phosphorylation of ERK1/2, PI3K p110α, and Akt not only in VSMCs isolated from normotensive rats but also in hypertensive rats. This may have clinical relevance in conditions associated with higher levels of Ang II and Ang II-mediated hyper-signaling of proliferation pathways in the CVS, such as in hypertension and atherosclerosis. Nonetheless, further in vivo studies are required to demonstrate the protective effects of oxytocin against deleterious effects of Ang II in these pathophysiological conditions.

The study findings further revealed that oxytocin and its pre-treatment with Ang II led to dysregulation of the ROCK pathway via inhibition of the activation of ROCK1 and ROCK2. These findings suggest a possible cardioprotective role of oxytocin in conditions where there is an excessive vasocontraction of blood vessels linked with Ang II-mediated hyper-signaling of ROCK pathway, for example, hypertension. However, this study was limited in its scope with regards to oxytocin's effect on vasocontraction as the study did not directly measure vasocontraction of VSMCs and rather investigated the vasocontraction pathway, and this could be further investigated in the future.

Furthermore, the results of an ELISA-based IL-6 assay indicated that oxytocin's treatment increased IL-6 secretion in aortic VSMCs. This may be beneficial in conditions where a short-term increase in IL-6 release bolsters immunological response against a pathogenic stimulus for a shorter duration. Also, our findings demonstrated that oxytocin reduced the Ang II-mediated IL-6 secretion in aortic VSMCs isolated from both Wistar rats and SHRs. This could be favorable in conditions associated with Ang II-induced prolonged inflammation such as rheumatoid arthritis. However, further studies investigating oxytocin's effect on inflammation and its interaction with Ang II on the inflammatory response, especially in vascular conditions associated with aggravated inflammation such as atherosclerosis, are required.

The ROS assay findings showed that oxytocin increased ROS production and its pre-treatment with Ang II further increased ROS production in aortic VSMCs isolated from both Wistar rats and SHRs. This suggests that oxytocin might

increase oxidative stress in cultured VSMCs. However, it cannot be assumed that in vivo, oxytocin would exhibit similar effects. In vivo, VSMCs co-exist with vascular endothelial cells, wherein oxytocin has been previously shown to reduce ROS production. It could very well be that the opposite effects of oxytocin on ROS production in VSMCs and endothelial cells cancel out each other and might not disturb the overall redox equilibrium. Henceforth, oxytocin might not contribute to a change in oxidative stress in blood vessels, which could be significant in pathophysiological conditions associated with increased oxidative stress in CVS. Nevertheless, further studies would be required where oxytocin's effect on ROS can be assessed simultaneously in both VSMCs and endothelial cells, such as in vitro studies employing co-culturing of VSMCs and endothelial cells and in vivo studies.

5.7. Conclusion

VSMCs undergo various pathophysiological changes in hypertension and other CVD, which render them as a potential target for newer approaches for their therapeutic management. The present in vitro study investigated the effects of oxytocin on cell proliferation, inflammation, oxidative stress, and also on the pathways involved in cell proliferation and vasocontraction in primary aortic VSMCs, isolated from normotensive (Wistar rats) and hypertensive rats (SHRs).

This study demonstrated that oxytocin inhibits proliferation and increases inflammation (IL-6) and oxidative stress (ROS) in aortic VSMCs obtained from Wistar rats and SHRs. Based on the study outcomes, it can be suggested that the anti-proliferative effect of oxytocin is mediated by inhibition of ERK1/2 and PI3K/Akt phosphorylation. Also, oxytocin deactivated Rho-kinase signaling pathway, a critical pathway involved in the contraction of VSMCs, by inhibiting the phosphorylation of ROCK-1 and ROCK-2.

The increased proliferation in VSMCs and hypercontraction mediated by peptides such as Ang II, worsen hypertension and precipitate other CVD and their progression. Oxytocin not only decreased VSMCs proliferation but also reduced Ang II-induced VSMCs proliferation and Ang II-stimulated vasocontraction pathways.

Overall, this study indicates that oxytocin might have therapeutic potential in the management of CVD including hypertension. However, future in vivo studies are required to examine the cardiovascular protective effect of oxytocin in hypertension.

5.8. Future recommendation

The present study utilized all the existing techniques available to assess the effect of oxytocin and its pretreatment with Ang II on cell proliferation in VSMCs. However, the study was limited in its scope with regards to the direct measurement of vasocontraction in aortic VSMCs.

The current study indirectly assessed vasocontraction by investigating the vasocontraction pathway — ROCK pathway. In the future, this aspect can be addressed. The effect of oxytocin on vasocontraction can be studied by determining the intracellular calcium levels in aortic VSMCs. This can be achieved by employing techniques such as fluorescent-based calcium measurement assay and more directly employing organ bath studies using vascular smooth muscle tissue.

Furthermore, to understand the mechanism of how oxytocin decreases the phosphorylation of the ERK1/2, PI3K p110α, and Akt, $G\alpha_i$ inhibitors may be employed. Since oxytocin receptors are GPCRs and have been previously shown to recruit different G-proteins in different cells and in different physiological conditions, it is a likely that oxytocin recruits $G\alpha_i$ and thus inhibits ERK1/2 and PI3K/Akt-mediated downstream signaling pathways involved in the proliferation of VSMCs.

Another such possibility could be that oxytocin stimulates phosphatases and consequently inhibits the phosphorylation of ERK1/2, PI3K p110α, and Akt. This can be explored in future research endeavors.

Also, the present study employed in vitro experimental models. All the study experiments were performed on primary aortic VSMCs isolated from Wistar

rats and SHRs. This study has generated sufficient scientific evidence to warrant the use of in vivo experimental models in the future. And, based on the results of a future in vivo study, the next step would be to conduct a pilot clinical trial as oxytocin is a peptide that has been approved by the food and drug administration (FDA), and has been subjected to various clinical trials.

As demonstrated by the study outcomes, this research highlighted the significant vascular protective effects of oxytocin and its antagonistic actions on Ang II effects in VSMCs isolated from both normotensive and hypertensive rats. These findings suggest that oxytocin may have therapeutic implication in conditions where hyper-activation of Ang II leads to hyper-signaling of Ang II-mediated signaling pathways resulting in the development and progression of various CVD, including hypertension. However, future in vivo studies and clinical studies would be required to explore the protective effect of oxytocin in CVD.

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